SYNTHESES OF POSSIBLE STEROID INTERMEDIATES IN BILE ACID BIOSYNTHESIS

By

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SUMMARY

The structural relationship of cholesterol and bile acids was established on a chemical basis as early as the beginning of the 20th Century by Windaus. (For a review see "Steroids" by Fieser and Fieser.) But the first direct evidence that cholesterol was a biological precursor of bile acids was reported in 1943 (Bloch et.al J.Biol.Chem. 149, 511). Since then, many studies on the degradation of cholesterol to bile acids have been made. These studies require postulated synthesized intermediates for biological testing, therefore the chemical synthesis of hypothetical intermediates is important. The main pathway for the biogenesis of cholic acid possibly involves initial hydroxylation of cholesterol at the 7α-position, followed by oxidation of the 3β-hydroxyl group to give cholest-4-en-3-one-7α-ol. The 12α-hydroxyl group is probably then inserted and the molecule then reduced to the 5β-cholestan-3α,7α,12α-triol. This is followed by ω-oxidation of the side chain and then β-oxidation and cleavage to cholic acid. The subject has been reviewed in this work.

The aim of the present work was to produce a series of cholesterol derivatives which may be intermediates in cholesterol metabolism in living cells. In certain cases newer methods for the synthesis of known sterols were evolved. Methods for the improved separation of certain sterols had also to be worked out, and modern methods for criteria of purity were examined.

The 7α-hydroxy derivatives of cholesterol, 12α-, 24, 25 and

26-hydroxycholesterols - the triols, were prepared through the respective 7α-hydroperoxides by means of photosensitized oxygenations. Studies with the cholesterol-4-C^{14} revealed that with either very dilute solutions or concentrated solutions, the attack of molecular oxygen was not as highly specific as often stated. The stereospecificity of the mode of attack of molecular oxygen has been reviewed. The isolation of cholest-4-en-3β,6β-diol in photosensitized oxygenation of cholesterol is reported and its mode of formation discussed.

It is known that the 12α-hydroxylation reaction does not occur after either the 26-hydroxylation has been effected or the side chain oxidised to a C_{24}-acid. Certain novel compounds such as cholest-5-en-3β,7α,24ξ-triol and cholest-5-en-3β,7α,25-triol, are in the course of tritiation and the in vitro studies will be of interest to ascertain whether they are converted into cholic acid.

The substance 7β-hydroxycholesterol was prepared by the $NaBH_4$ reduction of 7-ketocholesterol. Separation of the 7α- and 7β-epimers was achieved by chromatography of the diacetate as well as the free diols on neutral alumina. For successful separation of the free diols the amount of water in the alumina was critical. No separation was possible when no water was used, while the best separation was obtained with 1% water in the support.

The sterol, 12α-hydrocholesterol was prepared by a modified method of Danielsson (1961b). [illegible] Kemi (1961) 17, 381).

Deoxycholic acid was coupled with isovaleric acid, and 5β-cholestan-3α,12α-diol thus obtained was oxidised to the 3-oxo compound by an Oppenauer method. The 4,5-double bond was introduced using SeO_2 in ethanol and cholest-4-en-3-one-12α-ol obtained in good yields. The reported method of Danielsson involved a 3-stage synthesis for the introduction of a 4,5-double bond, and the yields were low. The reduction of the enol-acetate of the α,β-unsaturated ketone with $NaBH_4$ gave the desired product. The working up procedure was also modified, and a new compound cholest-3,5-diene-12α-ol was isolated and identified as a side product.

Another new compound i.e. 5β-cholestan-3α,12α-diol-24-one was prepared by utilizing the known reaction of an acid chloride and di-isopropyl-cadmium. This compound could be used to prepare cholest-5-en-3β,12α,24ξ-triol using the sequence of reaction described previously. Another interesting biological compound, cholest-5-en-3β,7α,12α,24ξ-tetrol, can be obtained from this triol on photo-oxygenation and reduction as described in this work.

The 4β, 22ξ, 24, 25, 26-hydroxycholesterols were prepared by modifications of the reported methods and 26-hydrocholesterol was prepared by two different routes. An impurity separated from 25-keto-nor-cholesterol acetate was identified as 3β-acetoxy-20-hydroxy-5-cholenic acid lactone.

Cholestan-3β,5α,6β-triol, cholestan-3β,5α-diol-6-one, 5α,6α-epoxycholesterol, cholest-6-en-3β,5α-diol and cholestan-

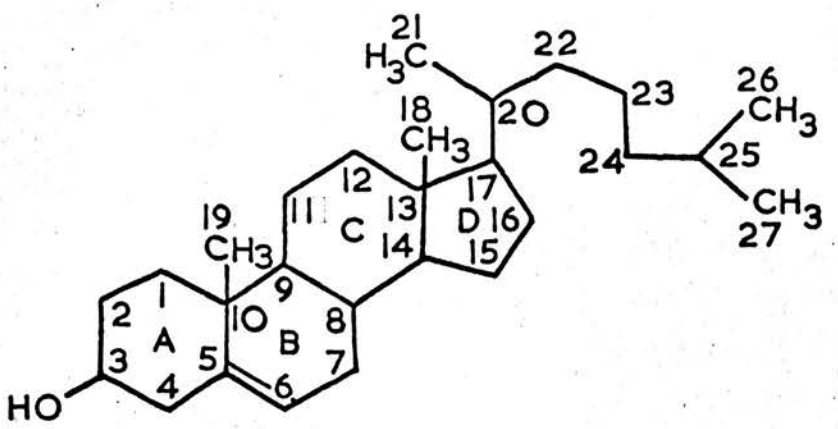
3β,5α-diol were prepared essentially by known methods.

Cholest-4-en-3-one-7α-ol was prepared by a known sequence but modification was made in the preparation of cholest-4,6-diene-3-one by the use of chloranil in the dehydrogenation of cholest-4-en-3-one.

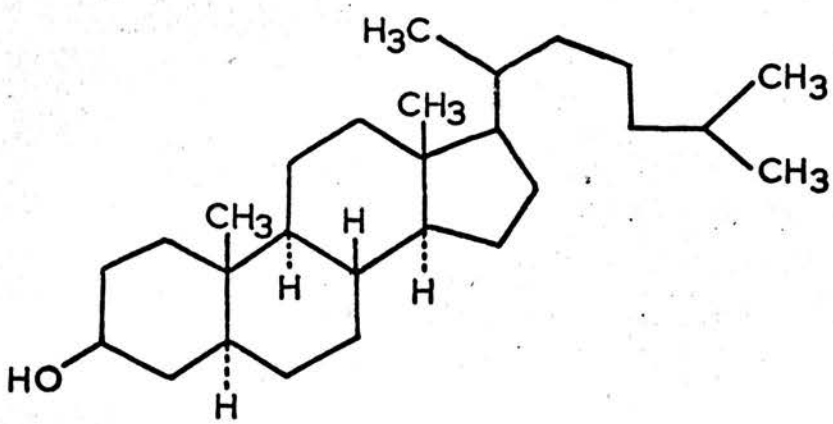
A number of intermediates involved in the inversion of the configuration at C_3 and the saturation of the double bond were synthesized. The starting materials for these compounds were saturated coprostanes, which were prepared by electrolytic coupling of the respective bile acids and isovaleric acid.

However, there are still gaps in our knowledge of complete sequence of the degradation of cholesterol to the bile acids. A comprehensive understanding of the subject will only be possible when all the relevent hypothetical intermediates have been synthesized and are available for biological experiments.

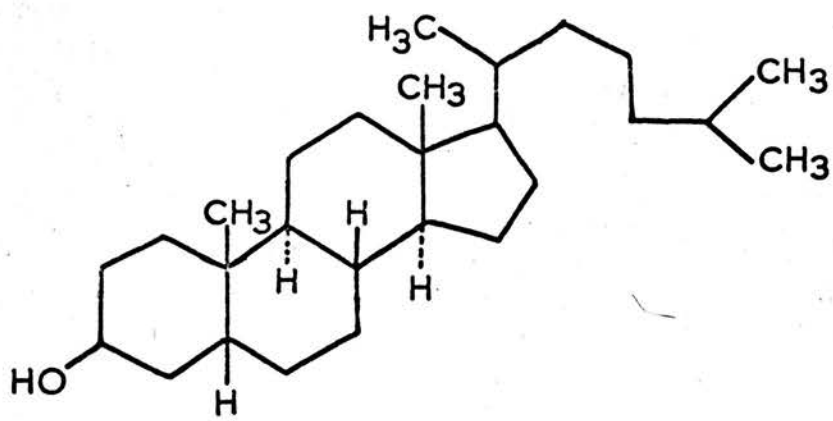
INTRODUCTION



CHOLESTEROL



CHOLESTANOL A/B trans, allo series.



COPROSTANOL A/B cis, normal series.

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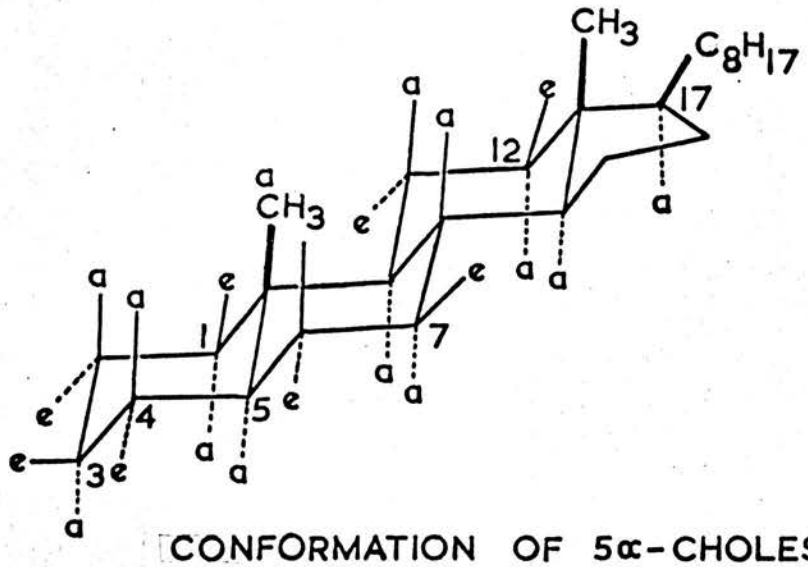
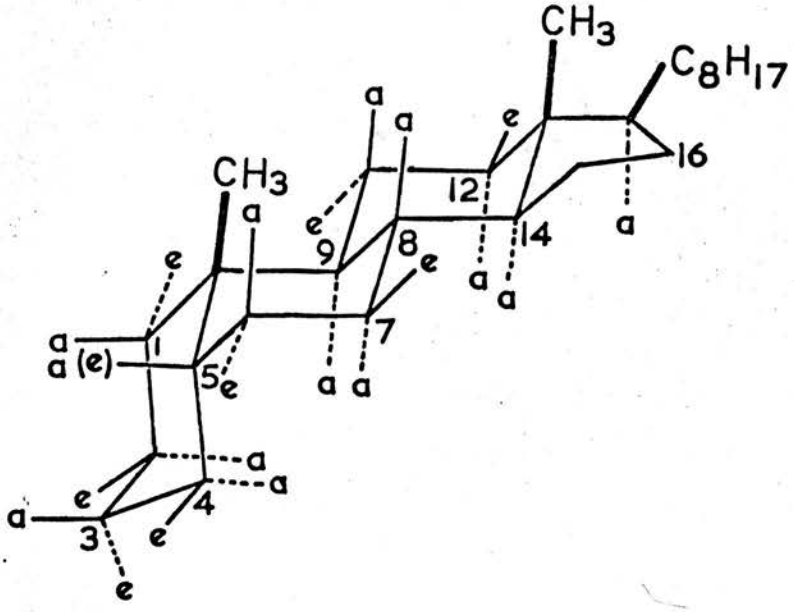


CONFORMATION OF 5α-CHOLESTANE

fig. 2 a.



CONFORMATION OF 5β-CHOLESTANE

fig. 2 b.

SECTION 1.

INTRODUCTION

The steroids form a group of structurally related colourless, crystalline compounds which are widely distributed in animals and plants. They all possess a phenanthrene ring system with an additional five membered ring attached to the 1,2 position, giving rise to a cyclopentanophenanthrene skeleton.

The unsaponifiable fraction of oils and fats contain among other chemical compounds a class of crystalline substances called sterols, which are C_{27} - C_{29} secondary alcohols possessing the characteristic perhydro-1,2-cyclopentanophenanthrene ring system. Cholesterol is the predominant mammalian sterol. The present day formulation of its structure is based on the classical researches of Wieland, Windaus, Diels, Rosenheim and King (review by Fieser and Fieser 1959). The stereochemistry and nomenclature of steroids are reviewed by Klyne (1957) and Shoppee (1964). (See figs. 1, and 2)

Cholesterol has been found in all tissues and is the principal constituent of gall stones deposited from bile. The 3β-hydroxyl group in cholesterol is alcoholic in nature and in all tissues except adult nervous tissues it is sometimes esterified with a variety of fatty acids. For example, in liver, cholesterol is esterified with poly-unsaturated fatty acids such as linoleic, oleic, and to a lesser degree with saturated acids like palmitic acid. The esters of short chain fatty acids do not occur in any

significant amounts.

The adrenal cortex, ovary and testis of most vertebrates are rich in cholesterol esters, liver, kidney and lungs are intermediate whereas bone, muscular tissue and cartilage are low in sterol esters. In adult nervous tissue there is a large amount of sterol, but it exists in the non esterified or "free" form, except in the developing brain where cholesterol is present in the esterified form. Cholesterol accounts for approximately 10% of the dry weight of brain in contrast to less than 1% found in most other organs (Davison 1965). Cholesterol is present in all parts of cells, bound to lipo-protein in the form of a complex, which appears to be an integral part of cell membrane.

Bile Acids

Bile acids are hydroxy derivatives of cholanic acid with a cis-A/B ring junction. They possess a carboxyl group attached to C_{23} and the number and position of the hydroxy groups vary in different bile acids. In general all the natural bile acids possess one hydroxyl group at the 3α-position. Other positions where hydroxyl groups may occur are carbon atoms 6,7,12 and 23.

Modern vertebrates which are more advanced than amphibians have C_{24}-bile acids. These bile acids are produced in the liver mainly by degradation of cholesterol and occur in bile as water soluble sodium salts of peptide conjugates with glycine or taurine. These conjugates or bile salts promote the

absorption of fats and water insoluble substances in the intestinal tract by acting as emulsifying agents and by facilitation of certain enzymic hydrolyses and re-esterifications.

Cholic acid (3α,7α,12α-trihydroxycholanic acid) and chenodeoxycholic acid (3α,7α-dihydroxycholanic acid) are regarded as 'primary' bile acids in mammals. Deoxycholic acid (3α,12α-dihydroxycholanic acid) and lithocholic acid (3α-hydroxycholanic acid) have been shown to be formed from cholic acid and chenodeoxycholic acid respectively by the action of the intestinal bacteria. These bile acids return to the liver during the entero-hepatic circulation, where further 7α-hydroxylation occurs in some species (Norman and Sjoval 1958) or 16α-hydroxylation as in Boidae (Haslewood 1967) may occur in the case of deoxycholic acid. In the case of lithocholic acid such hydroxylations may take place at positions 6 or 7 or both giving rise to hyocholic acids or α and β muricholic acids. Evidence for a pathway suggesting the formation of lithocholic acid as a primary bile acid has been recently advanced by Mitropoulos and Myant (1967).

Although most of the bile acids present in the gut are reabsorbed into the blood and transported to the liver during the entero-hepatic circulation a small quantity is excreted in the faeces with the result that new bile acids are constantly being synthesised to compensate for daily losses.

Pathways of biological degradation of cholesterol leading

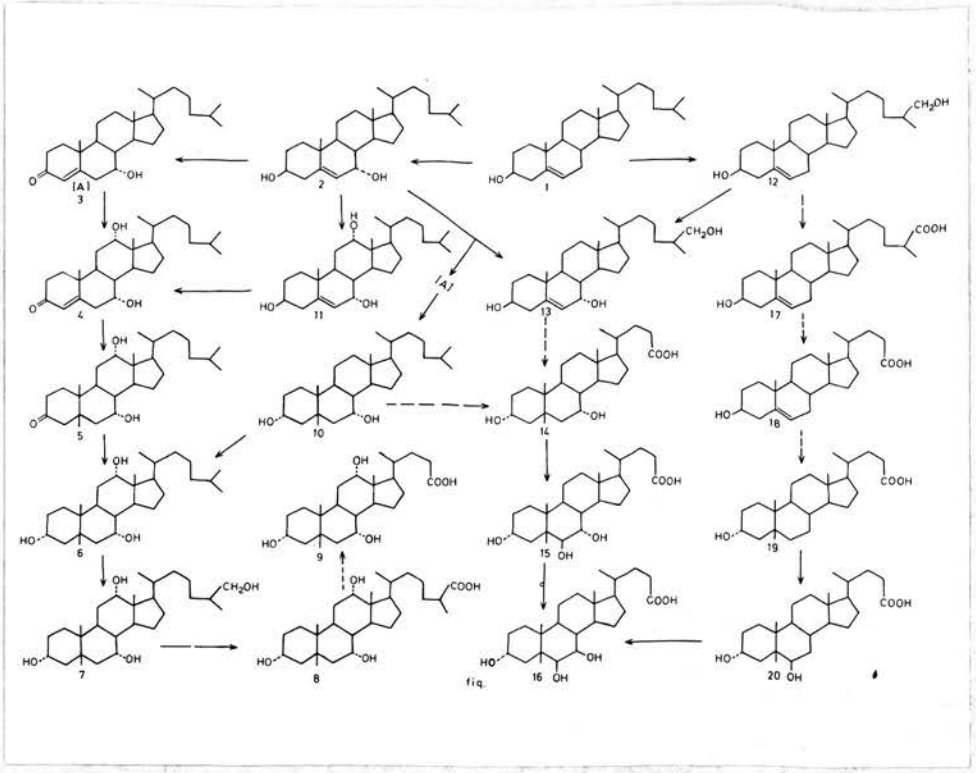


Fig. 3.

Pathways representing the known biological degradation of cholesterol to different bile acids.

1, Cholesterol; 2, Cholest-5-en-3β,7α-diol; 3, Cholest-4-en-3-one -7α-ol; 4, Cholest-4-en-3-one-7α,12α-diol; 5, 5β-cholestan-3-one-7α,12α-diol; 6, 5β-cholestan-3α,7α,12α-triol; 7, 5β-cholestan-3α,7α,12α,26-tetrol; 8, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; 9, Cholic acid; 10, 5β-cholestan-3α,7α-diol 11, Cholest-5-en-3β,7α,12α-triol; 12, Cholest-5-en-3β,26-diol; 13, Cholest-5-en-3β,7α,26-triol; 14, Chenodeoxycholic acid; 15, α-muricholic acid; 16, β-muricholic acid; 17, 3β-hydroxy cholest-5-enoic acid; 18, 3β-hydroxychol-5-enoic acid; 19, Lithocholic acid; 20, 3α,6β-dihydroxy-5β-cholanoic acid.

to the formation of bile acids and steroid hormones have been suggested. The catabolism of cholesterol to these compounds is physiologically important. The intermediates involved in the stepwise degradation of cholesterol have not been fully elucidated, but they seem to involve certain stages in which there is the interaction of molecular oxygen with cholesterol forming various hydroxylated and oxygenated derivatives.

DEGRADATION TO BILE ACIDS.

Bloch, Berg and Rittenberg (1943) and then Fukushima and Gallagher (1952) demonstrated that cholesterol is the precursor of bile acids when they administered by intravenous infusion deutero-cholesterol to a dog whose bile duct was connected to the kidney and obtained labelled cholic acid from the urine.

Similar experiments using cholesterol-4-C^{14} on rabbits (Ekdahl and Sjovall 1955) and rats (Bergstrom and Norman 1953) demonstrated the conversion of cholesterol to physiological bile acids.

Theoretically, therefore, four structural changes in the cholesterol molecule must take place in order that cholesterol is converted to bile acids. These changes can be summarised as under:

(a) Epimerisation of 3β-hydroxy group to 3α-hydroxy group.

(b) Saturation of the 5,6 double bond to give rise to 5β or cis-series (coprostane type) of compounds.

(c) Hydroxylation at 7α or 12α positions or both.

(d) Oxidation of the side chain to give a terminal C_{24} carboxylic acid.

Since the exact sequence of changes in structure leading to the biosynthesis of bile acids from cholesterol are not known, therefore by permutation and combination the number of possible intermediates is very great. One of the recent experimental approaches involved in solving the mechanism of degradation of cholesterol to bile acids, is to study the metabolism of different hypothetical intermediates both *in vivo* and *in vitro*.

I. CATABOLISM OF CHOLESTEROL TO BILE ACIDS.

A. NUCLEAR CHANGES

According to the recent view as to how cholesterol is degraded to cholic acid *in vivo*, saturation of the 5,6-double bond, epimerization of the 3β-hydroxyl group and hydroxylation at positions 7α and 12α take place before oxidation and cleavage of the side chain occurs. The main evidence for this concept is based on the fact that experiments with labelled cholest-5-en-3β,7α-diol (Bergstrom and Lindstedt 1956) and labelled 5β-cholestan-3α,7α,12α-triol (Bergstrom, Paabo and Rumpf 1954) using the bile-fistula technique resulted in the conversion of these compounds to cholic acid, whereas cholest -5-en-3β,26-diol (Danielsson 1961) or 3β-hydroxychol-5 -enoic acid (Bergstrom 1955) were not converted to cholic acid.

3β-Hydroxy-chol-5-enic acid was however found to be hydroxylated at position 7 (Usui and Yamasaki 1960) but the configuration of the newly introduced group was not ascertained. Later on it was found that both 7α and 7β-hydro-

xylases, of 3β-hydroxychol-5-enic acid (Yamasaki et.al.1966) are present in liver microsomes. Therefore it is reasonable to assume that whereas in the formation of chenodeoxycholic acid, 7α-hydroxylation could be effected, 12α-hydroxylation for/ the biosynthesis of cholic acid does not proceed after modification of the side chain (Danielsson 1963; Mitropolous and Myant 1967).

(i) EVIDENCE FOR 7α-HYDROXYLATION.

In order to ascertain whether epimerisation of the 3β-hydroxyl group and saturation of 5,6-double bond precede the introduction of/ the 7α-hydroxy or 12α-hydroxy or both in the steroid nucleus, the following hypothetical intermediates in the degradation of cholesterol to bile acids were tried in bile-fistula rats by Harold, Jayko and Chaikoff (1955); Harold, Chapman and Chaikoff (1957) and Bergstrom (1955). (a) Cholest-5-en-3-one, (b) Cholest-5-en-3αol, (c) Cholest-4-en-3-one, (d) 5β-cholestane-3β-ol, (e) 5β-cholestane-3α-ol.

Of these mono-oxygenated C_{27}-steroids none but cholesterol gave rise to cholic acid, indicating that epimerisation and saturation of the double bond must occur after α-hydroxylation at C_7 or C_{12} or both. This conclusion obtained support from the later investigations of Bergstrom and Lindstedt (1956) and then Lindstedt (1957) who studied the labelled products excreted in the bile of cannulated rats, after administration of isotopically labelled cholest-5-en-3β, 7α-diol and 5β-cholestan-3α,7α-diol. Both these sterols were

converted to chenodeoxycholic acid and cholic acid; cholest-5-en-3β,7α-diol being a better intermediate in the formation of cholic acid. On the basis of these experiments Bergstrom and Lindstedt concluded that the introduction of an α-hydroxyl group at position 7 is an early step in the formation of both cholic acid and chenodeoxycholic acid, and cholest-5-en-3β,7α-diol probably being an early intermediate (also Mendelsohn and Staple 1963).

This suggestion seems to be in accordance with the chemical aspect because the 7 position is very active due to the double bond at the 5-6 (allylic) position as shown by the ease of oxidation at that position.

The stereo-chemical mode of 7α-hydroxylation was investigated by Bergstrom, Lindstedt, Samuelson, Corey and Gregoriou (1958). These workers studied the conversion of cholesterol stereospecifically labelled with tritium in the 7α and 7β positions, and found that the 7-hydroxyl group specifically replaced the 7α-hydrogen with essentially complete retention of configuration leaving the 7β-hydrogen intact. Thus the possibility of hydroxylation at the 7α-position by hydration of an olefinic intermediate or a 7-keto intermediate is completely eliminated.

The actual identification of 7α-hydroxycholesterol as a biological intermediate was shown in 1964 when Danielsson and Einarsson isolated enzymically formed labelled 7α-hydroxycholesterol from labelled cholesterol in their studies on rat liver

homogenates. They also reported that the 7α-hydroxylase was microsomal. Mendelsohn, Mendelsohn and Staple (1965) also confirmed the enzymic formation of both 7α- and 7β-hydroxy-cholesterols in their in vitro experiments.

Rat liver is capable of 7α-hydroxylation of taurodeoxy-cholic acid, but this enzyme is different from the other 7α-hydroxylase which requires cholesterol as substrate. Deoxycholic acid in this animal as well as in the rabbit and man is known to be formed from cholic acid by the action of intestinal bacteria and enters the liver via the entero-hepatic circulation. Therefore such formation of cholic acid from deoxycholic acid cannot be regarded on the line of formation of primary bile acids in the rat.

II. EVIDENCE FOR 12α-HYDROXYLATION.

The order in which hydroxylations at 7 and 12 positions take place during the formation of cholic acid appears to be established by the fact that cholest-5-en-3β,7α-diol is converted in good yield to cholic acid in the bile fistula rat, cholest-5-en-3β,12α-diol on the contrary is converted less efficiently to cholic acid in the bile fistula rabbit (Danielsson 1962; see also Danielsson 1961[b]).

Also in their in vitro studies of conversion of cholesterol to 5β-cholestan-3α,7α-diol and 5β-cholestan-3α, 12α-diol, Mendelsohn, Mendelsohn and Staple (1965[a]) detected no significant activity in 5β-cholestan-3α,12α-diol used as a trapping agent after the incubation was performed. Such

treatment of the incubation mixture with 5β-cholestan-3α,7α-diol affords a distinctly active preparation. This suggests that 7α-hydroxylation of the steroid nucleus is a prerequisite for 12α-hydroxylase activity. Further cholest-5-en-3β,25-diol (Frederickson and Ono 1956) cholest-5-en-3 ,26-diol (Danielsson 1962) cholest-5-en-3β,7α,26-triol (Danielsson 1961[c]) 5β-cholestan-3α,7α,26-triol, (Berseus and Danielsson 1963) 5β-cholestan-3α,7α-diol-26-oic acid (Bridgewater and Lindstedt 1963) 3α,7α-dihydroxycholanic acid (Lindstedt and Sjovall 1957) in all of these compounds where the side chain is modified, they have been reported to give rise to mainly chenodeoxycholic acid, and the formation of cholic acid is very insignificant. On the other hand 3α,7α,12α-trihydroxycoprostanic acid is converted very efficiently to cholic acid both in the bile fistula rat and in liver homogenates (Bergstrom, Bridgewater and Gloor 1957). These observations clearly demonstrate that in the formation of cholic acid the introduction of the 12α-hydroxy group does not take place after any change which entails modification or elimination of the terminal isopropyl group in the side chain; however 6β hydroxylation may take place instead. (See figure 3 also.)

EVIDENCE FOR INVERSION OF CONFIGURATION AT 3 and 5 POSITIONS.

Yamasaki, Noda, and Shimizu (1959) incubated cholest-5-en-3β,7α-diol with a cell-free preparation from rat liver and on the basis of an increase in optical density at 240 mμ proposed that cholest-5-en-3β,7α-diol was oxidised to cholest-4-en-3-one-7α-ol, as an intermediate during conversion of 7α-hydroxy-

cholesterol to 5β-cholestan-3α,7α-diol. This suggestion implies the epimerisation of the 3β-hydroxy to 3α-hydroxy through the intermediate formation of a ketone.

Indeed when Rosenfeld and Hellman (1961) administered 3α-tritio-cholesterol to a patient with a bile fistula no radioactivity was found in the chenodeoxycholic acid and cholic acid obtained from the bile. Similar experiments by Samuelsson (1963) led him to believe that inversion of the 3β-hydroxy proceeds via a 3-ketone as tritium in the 3α-position of cholesterol was lost during formation of cholic acid in rat liver. The same author in 1959 demonstrated that the saturation of/the 5,6 double bond takes place after its isomerization to/the 4,5-position in a stereospecific manner by cis- addition from the β-side of the molecule as in the metabolism of certain steroid hormones. Green and Samuelsson (1964) on the conversion of 3α-^{3}H-cholesterol and 4β-^{3}H-cholesterol to bile acids in bile fistula rat also indicated that the transformation of the 5-ene-3β-ol configuration into 5β-3α-ol involved a 3-keto steroid with the 5,6 double bond isomerised to the allylic 4,5 position before reduction. A similar mechanism for the epimerisation of carditonic steroids at position 3 in rat liver has been proposed by Rapke and Samuelsson (1964). Very recently it has been shown that the biogenis of 5α-bile alcohols may also involve the formation of an α,β-unsaturated ketone for example the formation of 5α-Cyprinol (Hoshita 1967).

Danielsson (1961c) studied the metabolism of cholest-5-en-3β,7α-diol in mouse liver homogenates and identified cholest-4-en-3-one-7α-ol as one of the two main metabolites, the other one being assigned the structure of cholest-4-en-3-one-7α,26-diol on the basis of its mobility on thin layer chromatographic plates, the characteristic absorption of α,β-unsaturated ketone, and also that it was converted to chenodeoxycholic acid when injected into a bile fistula rat. Cholest-5-en-3β,7α,26-triol was also identified as a minor metabolite.

Hutton and Boyd (1966) in their <u>in vitro</u> experiments also obtained similar results. They found that the liver microsomal fraction was four times as efficient as the mitochondrial fraction in converting 7α-hydroxy cholesterol to cholest-4-en-3-one-7α-ol. They also suggested that cholest-4-en-3-one-7α,26-diol must have been formed from cholest-5-en-3β,7α,26-triol in mitochondria.

When tritium labelled cholest-4-en-3-one-7α-ol was injected into a bile fistula rat, Danielsson (1961d) found that the radioactivity was excreted in bile as bile acids and was distributed mainly in chenodeoxycholic acid and cholic acid. Several unidentified acids were also formed.

Hutton and Boyd (1966a) studied the metabolism of cholest-4-en-3-one-7α-ol with different cell fractions of liver and found that in mitochondria this compound was converted to cholest-4-en-3-one-7α-26-diol and in microsomes alone it

remained unchanged. They also reported that with the cell supernatant fraction cholest-4-en-3-one-7α-ol was reduced to cholest-4-en-3α,7α-diol and 5β-cholestan-3α,7α-diol. Similarly 5β-cholestan-3-one-7α-ol was converted readily to 5β- cholestan-3α,7α-diol whereas cholest-4-en-3α,7α-diol was not readily converted to 5β-cholestan-3α,7α-diol. Thus they proposed two possible routes for the formation of 5β-cholestan -3α,7α-diol from cholest-4-en-3-one-7α-ol depending upon whether the 4,5 double bond or the 3 ketone is reduced first.

There seem to be two schools of thought regarding the order in which 3-ketone and 4,5 double bond is reduced so that finally a 5β-3α-ol grouping is obtained. Danielsson (1961e) reported the conversion of 5β-cholestan-3-one-7α-ol to cholic and chenodeoxycholic acids, when administered intraperitoneally to a bile fistula rat and he favoured first reduction of the double bond leaving the 3-keto intact. Hutton and Boyd (1966a) studied the comparative ease of reduction of 5β-cholestan-3-one-7α-ol and cholest-4-en-3α,7α -diol and found that in vitro the former was readily and completely converted to 5β-cholestan-3α,7α-diol whereas the latter compound was only partially reduced to 5β-cholestan-3α, 7α-diol. Because 5β-cholestan-3-one-7α-ol was converted very readily to 5β-cholestan-3α,7α-diol (dihydroxycoprostane) it did not accumulate and therefore could not be detected. They favoured both routes in which case 5β-cholestan-3-one-7α-ol as well as cholest-4-en-3α,7α-diol would be intermediates.

Mendelsohn, Mendelsohn and Staple (1966) on the basis of *in vitro* experiments where they detected virtually no radioactivity in the area of coprostan-3-one-7α-ol in their assay on thin layer chromatography, maintained that in such experiments coprostan-3-one-7α-ol was not an intermediate. They also claimed that the 3-keto group would not reduce to the corresponding α-alcohol in the saturated keto steroid, and further proposed that reduction of the 3-keto group could take place just prior to or simultaneously with the reduction of the 4,5 double bond. In the first case cholest-4-en-3α,7α-diol would be an intermediate.

FURTHER EVIDENCE FOR 12α-HYDROXYLATION.

Theoretically there can be several possibilities of 12α-hydroxylation. Either it can take place just after 7α-hydroxylation i.e. formation of 7α-hydroxycholesterol in which case cholest-5-en-3β,7α,12α-triol will be an intermediate, (Berseus, Danielsson, Einarsson (1967) or after the oxidation of the 3β-hydroxyl group to a ketone with simultaneous allylic shift of the double bond to the 4,5 position when cholest-4-en-3-one-7α,12α-diol would be an intermediate (Danielsson and Einarsson 1966), and cholest-4-en-3-one-7α-ol would be the substrate for the 12α-hydroxylase. If 12α-hydroxylation takes place after complete reduction of the 4ene-3one grouping to the 3α-ol-5β grouping, in that case 5β-cholestan-3α,7α,12α-triol can be an intermediate and the substrate for 12α-hydroxylase would be 5β-cholestan-3α,7α-diol

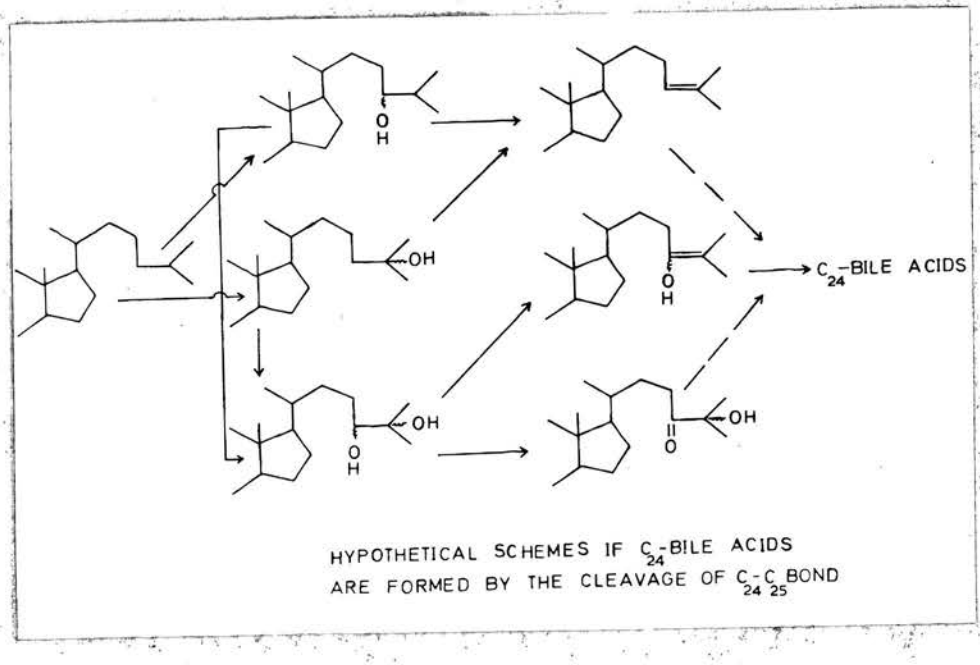
(Mendelsohn, Mendelsohn and Staple 1966). Should 12α-hydroxylation take place after partial reduction of the α,β unsaturated ketone the substrate for 12α-hydroxylase would be cholest-4-en-3α,7α-diol, cholest-4-en-3β,7α-diol or 5β-cholestan-3-one-7α-ol, depending whether the 3-ketone is reduced while the 4,5 double bond was intact or vice versa and hence cholest-4-en-3α,7α,12α-triol, cholest-4-en-3β,7α, 12α-triol and 5β-cholestan-3-one-7α,12α-diol would probably be intermediates in the formation of cholic acid. In <u>in vivo</u> experiments cholest-4-en-3β,7α,12α-triol and cholest-4-en-3α,7α,12α-triol have been converted to cholic acid (Bjorkhein and Danielsson 1965).

Both/the 12α-hydroxylase and the enzyme systems for dehydrogenation and isomerization of the 5-ene,3β-ol grouping to the corresponding α,β-unsaturated ketone are located in the endoplasmic reticulum of liver cells, while the reducing enzymes are present in the cytoplasm or cell sap. It would seem more logical to assume that a molecule such as cholest-4-en-3-one-7α-ol would be 12α-hydroxylated while in the endoplasmic reticulum before moving to the cell sap to be reduced to 5β-cholestan-3α,7α,12α-triol rather than shuttling between events in the endoplasmic reticulum and events in the cell sap.

Cholest-5-en-3β,7α-diol, cholest-4-en-3-one-7α-ol, cholest-4-en-3α,7α-diol and 5β-cholestan-3-one-7α-ol are all reported to be converted into cholic acid and chenodeoxycholic

acid in good yield therefore in vivo experiments on bile fistula animals do not provide sufficient information as to the identity of the physiological substrate for the 12α-hydroxylase system. This lack of selectivity by the 12α-hydroxylase may be attributed to the abnormal nature of such animals whose entero-hepatic circulation has been broken by bile duct cannulation, which causes enhanced liver hydroxylase activity when compared with the intact (normal) animals.

Whether 12α-hydroxylation occurs immediately after the formation of 7α-hydroxycholesterol or after the initiation of the set of reactions involved in the formation of cholest-4-en-3-one-7α-ol nevertheless cholest-4-en-3-one-7α,12α-diol would be an intermediate in the formation of cholic acid. In fact cholest-4-en-3-one-7α,12α-diol has been identified as one of the major metabolites formed from cholesterol, cholest-5-en-3β-7α-diol and cholest-4-en-3-one-7α-ol (Danielsson and Einarsson 1966).



HYPOTHETICAL SCHEMES IF C_{24}-BILE ACIDS
ARE FORMED BY THE CLEAVAGE OF C_{24}-C_{25} BOND

Figure 4.

B. EVIDENCE FOR THE STEPS IN SIDE-CHAIN OXIDATION.

Metabolic degradation of the side chain may occur either (a) by direct cleavage of carbon-carbon bond between C_{24} and C_{25} of the sterol molecule giving a C_{24} and a C_3 unit or (b) by gradual oxidation of the three terminal carbon atoms or (c) by ω-oxidation followed by β-oxidation.

The sequence of events in side chain cleavage is still open to speculations and the literature on this, points to at least three possible pathways. However, all the evidence points to the involvement of tetrols or pentols or both in the formation of cholic acid (Danielsson 1963). It is difficult to assess which of the three mechanisms in the main pathway for formation of the C_{24}-bile acids, but the recent studies favour ω-oxidation followed by β-oxidation.

If in the biogenesis of bile acids from cholesterol the side chain is cleaved between C_{24}-C_{25} bond, 3 hypothetical schemes (fig. 4.) can be drawn, and 24 or 25-hydroxy; 24,25-dihydroxy; 24-dehydro and 25-hydroxy-24-keto-derivatives could be possible intermediates. The cleavage of the last type i.e. substances with a $-\overset{OH}{\overset{|}{C}}-\overset{O}{\overset{||}{C}}-$ grouping is a reaction well established in steroid biochemistry as examplified by the degradation of C_{21} to C_{19} steroid hormones.

In the formation of 24,25 dihydroxy derivative it is probable that 25-hydroxylation could be the primary step followed by 24-hydroxylation since tertiary carbon atoms are known to be more reactive than secondary or primary

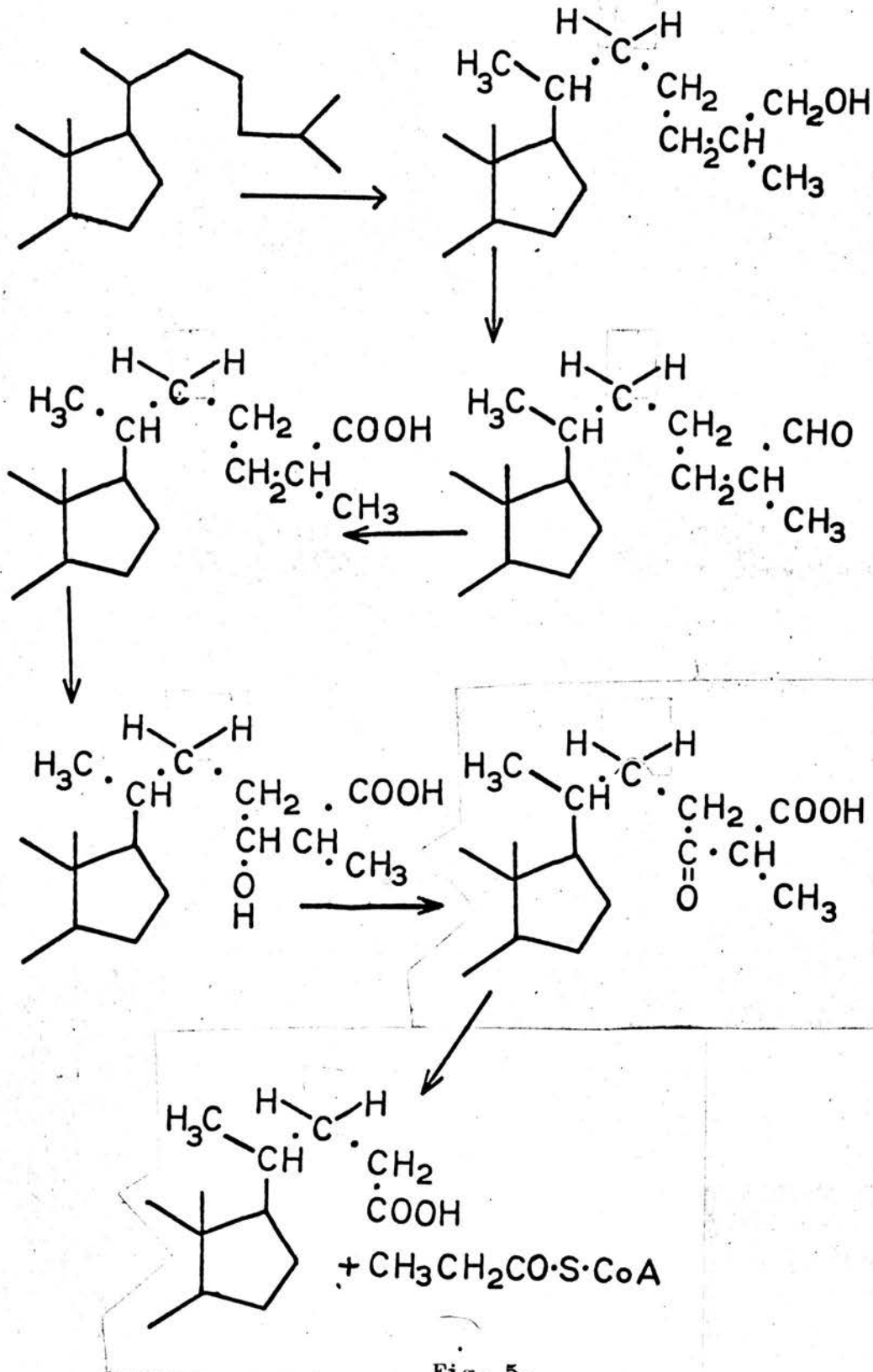


Fig. 5.

carbon atoms towards oxidation. Also by analogy with the mechanism of cleavage of the cholesterol side chain in adrenal mitochondria yielding pregnenolone, where 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol are regarded as possible intermediates. The reported formation of acetone by the cleavage of the C_{24}-C_{25} bond suggests that there may be more than one pathway (Whitehouse et.al 1960 and 1961). In the oxidation of the side chain the initial hydroxylation could occur at C_{26} or C_{27} followed by further oxidation to a carbonyl derivative. Either this compound would decarboxylate giving rise to a C_{26}-steroid and this could ultimately provide the desired C_{24}-bile acid. Alternatively by ω-oxidation of the side chain which involves 26 or 27-hydroxylation as an intermediate step the molecule may undergo β-oxidation to a C_{24}-bile acid as represented in fig. 5 . This mechanism appears to be established by the work of Danielsson (1960); Suld et.al (1962); Masui et.al (1966); Okuda and Danielsson (1965); and Mitropoulos and Myant (1956).

As early as 1953 Anfinsen and Horning described a mitochondrial system capable of oxidizing labelled cholesterol to CO_2. Fredrickson (1956) and Frederickson and Ono (1956) using the same mitochondrial system identified 25-hydroxy and 26-hydroxy-cholesterol in the non-volatile products in addition to acidic products of unknown nature.

There is now evidence that the first step in the

degradation of the side chain is the hydroxylation at position 26. Various 26-hydroxylated steroids have been isolated in in vivo and in vitro studies the most important being 26-hydroxycholesterol (Danielsson 1961a; see also Van Lier and Smith 1967) 5β-cholestan-3α,7α,12α,26-tetrol (Danielsson 1960) cholest-5-en-3β,7α-26-triol (Danielsson 1961a,c.) and cholest-4-en-3-one-7α-26-diol (Danielsson 1961c; Hutton and Boyd 1966a). It looks as if in higher vertebrates the 26-hydroxylation is stereospecific for example in the mouse (Berseus 1965) alligator and human where the 25α-isomer of 3α,7α,12α-trihydroxy-5β-cholestanoic acid is found (Carey and Haslewood 1963). On the other hand both 25α and 25β isomers of 3α,7α,12α-trihydroxy-5β-cholestanoic acid have been isolated from frog bile (Kurauti and Kazuno 1939; Mabuti 1941).

The further metabolism of 26-hydroxy derivative entails oxidation to the 5β-cholestanoic acid derivative thus effecting completion of the ω-oxidation. The substance 3α,7α,12α-trihydroxy-5β-cholestanoic acid has been isolated from mouse and rat liver homogenates, (Berseus, Danielsson and Kallner 1965) and from other vertebrates including human (Staple and Rabinowitz 1962). This C_{27}-acid has been reported to be converted to cholic acid both in vivo and in vitro (Briggs, Whitehouse and Staple 1961).

After completion of the ω-oxidation, there occurs β-oxidation and cholic and propionic acids are formed (Suld,

Staple and Gurin 1962).

The contention that the side chain hydroxylations resulting in the formation of tetrols or pentols in the biogenesis of C_{24}-bile acids especially cholic acid is supported by the work of Japanese workers on the less evolved vertebrates where the ultimate change from cholesterol is to C_{27}-bile alcohols. These bile alcohols have essentially the same nuclear part as found in cholic acid, but differ in retaining an intact side chain. The formation of bile alcohols may represent an "older type" of cholesterol metabolism which lacked certain more complicated or evolved enzymes as are present in higher vertebrates capable of utilizing these intermediate (bile alcohols) for further degradation to C_{24}-bile acids. The importance of these bile alcohols in the evolutionary tree, such as chimaerol (3α,7α,12α,24ξ,26-pentahydroxycoprostane); 3α,7α,12α,26-tetrahydroxycoprostane, 3α,7α,12α,25,26-pentahydroxy-Δ^{C}-coprostane (Enomoto 1962) and 5β-ranol (Betsuki 1966) and their acid derivatives such as 3α,7α,12α-trihydroxycoprostanic acid, and 3α,7α,12α,24ξ-tetrahydroxycoprostanic acid (Betsuki 1966) are important in this concept of the degradation of cholesterol to bile acids. In some animals secreting C_{27}-bile alcohols or C_{27}-bile acids a trace of cholic acid has also been detected. This may be regarded as the start of the enzyme systems present in higher animals where C_{24}-bile acids are the principal product of cholesterol metabolism. Some of

these C_{27}-bile alcohols and C_{27}-bile acids have been shown to be intermediates in bile acid biosynthesis in rats for example. 5β-ranol has been reported to be converted efficiently to cholic acid in bile fistula rats (Danielsson and Kazuno 1964).

Although 26-hydroxylation is a mitochondrial event, cytoplasmic enzymes are also required for the degradation of the side chain so that C_{24}-bile acids are produced (Danielsson 1960a, Mendelsohn, Mendelsohn and Staple 1965b). Since transformations in the side chain take place after events in the nuclear part of the molecule have taken place in the endoplasmic-reticulum, therefore the substrate for 26-hydroxylation could either be 5β-cholestan-3α,7α,12α-triol or cholest-4-en-3-one-7α,12α-diol for the formation of cholic acid and 5β-cholestan-3α,7α,12α-triol has long been regarded as an intermediate for 26-hydroxylation. In order that cholest-4-en-3-one-7α,12α-diol could be the substrate it would be necessary for the mitochondria to lie in a favourable juxtaposed position with the endoplasmic reticulum so that the molecule in question does not come in contact with the reductases present in the cytoplasmic part of the cell while moving from the endoplasmic reticulum to the mitochondria. Bjorkhem and Danielsson (1965) studied the metabolism of cholest-4-en-3α,7α,12α-triol and cholest-4-en-3β,7α,12α-triol and found that both were transformed efficiently into cholic acid in the bile fistula rat, and that the formation of cholic

acid occurred mainly through a 3-keto-steroid. Their results also indicate that cholest-4-en-3α,7α,12α-triol might be transformed into cholic acid to some extent through a pathway involving direct saturation of the 4,5 double bond.

In the light of experimental evidence obtained so far by various workers the sequence in the breakdown of cholesterol to C_{24}-bile acids the following steps seem to occur in order:

(1) The nucleus of cholesterol is hydrogenated and extra α-hydroxyl groups at position 7 or 12 (or both) are inserted.

(2) The end of the side chain is oxidized to give alcohols (C_{27}) and then acids (C_{27}).

(3) The side chain is shortened so that a C_{24}-bile acid is produced.

In some cases part of step (2) may occur before step (1).

There is suggestive evidence that during embryonic development every higher animal traces back its ancestry. The morphological recapitulation in this connection is highly fascinating. If during the differentiation of foetal or embryonic liver the enzymological developments also follow the evolutionary conception, then the catabolism of cholesterol with respect to bile acid formation at different stages may offer valuable information, especially in so far as the intermediates in the side chain degradation of cholesterol are concerned. In other words it is just possible that at

a certain stage of development the degradation of cholesterol may not reach the ultimate formation of C_{24}-bile acids, but possibly stop short at an early stage.

II CATABOLISM OF CHOLESTEROL TO STEROID HORMONES.

Of great physiological importance is the catabolism of cholesterol to the steroid hormones, which takes place in the endocrine tissues. The 20α-hydroxylation is regarded as the initial step in the cleavage of the side chain of cholesterol to give pregnenolone, and isocaproic aldehyde is also formed (Constantopolous and Tchen 1961). When labelled cholesterol was incubated with adrenal homogenates Solomon et.al (1956) were able to isolate labelled 20α-hydroxycholesterol, and no radioactivity was found in 22α-hydroxy, 22β-hydroxy, or 22-keto-20α-hydroxy-cholesterols. Since 20α-hydroxycholesterol was more efficiently converted to pregnenolone than cholesterol (Shimizu, Hayano, Gut and Dorfman 1961) by adrenal homogenates, this suggested that the 20α-hydroxylating system was required for pregnenolone formation. The further transformation of 20α-hydroxy-cholesterol may be through a labile 20α-22ξ-dihydroxycholesterol. Later these workers were able to identify 20α,22ξ-dihydroxycholesterol as an intermediate. Although the isomeric 22-hydroxycholesterols (Chaudhuri, Harada, Shimizu, Gut and Dorfman 1962) as well as cholest-5-en-3β,20α,-diol-22one (Constantopoulos, Carpenter, Satoh and Tchen 1966) have also been reported to be converted to pregnenolone in good yields this

does not eliminate the possibility of 20α,22ξ-dihydroxy-cholesterol formation. Formation of cholest-5,20(22)-diene, 3β-ol can also be envisaged in the former case. Simpson and Boyd (1967) could not detect the formation of 20α,22ξ-dihydroxycholesterol or 20α-hydroxycholesterol in adrenal mitochondrial preparations. However they isolated an intermediate, having the mobility between cholesterol and 20α-hydroxycholesterol. On reduction with $LiAlH_4$ this compound was reduced to a more polar product having the same mobility on TLC as 22ξ-hydroxycholesterol. The possibility that this compound could be a hydroperoxide was also eliminated, as it was not reduced to a diol with acidified KI solution in alcohol, and hence it might be 20,22-epoxycholesterol.

In the foregoing account it was pointed out that in the degradation of cholesterol to bile acids in the liver 7α-hydroxylation of cholesterol is an early event. There is a possibility that this hydroxylation is accomplished by an intermediate formation of a hydroperoxide, which could then be cleaved reductively in the presence of an electron donor to furnish the hydroxylated derivative. Although the formation of a 7α-hydroperoxy derivative has not been demonstrated in biological hydroxylation reactions nevertheless the hypothesis remains attractive in view of the fact that there seems to be a marked similarity between the mechanism of photochemical oxidation and biological hydroxylations. Further, almost all of the 'non-enzymic' products encountered in the biochemical in vitro work, namely cholest-5-en-3β-ol-7-one, cholest-5-en-3β,7β-diol, cholest-5-en-3β,7α-diol and cholestan-3β,5α,6β-triol are also either directly formed in the photo-oxygenation reaction mixture or can be predicted to be formed from the break-down products of the primary hydroperoxides. For example cholest-5-en-3β-ol-7α-hydroperoxide is known to decompose to cholest-5-en-3 -ol-7-one. Experimental evidence suggests that such a hydroperoxide can epoxidize cholest-5-en-3β-ol to give 5,6 epoxy-cholesterol while the hydroperoxide is reduced to cholest-5-en-3β,7α-diol analogous to the epoxidation reactions of olefins by peracids. Hence cholest-5-en-3β,7α-diol may arise directly and not through the reduction of cholest-5-en-3β-ol-7-one, which would give mainly cholest-5-en-3β,7β-diol.

The 5,6 epoxy-cholesterol on hydrolytic cleavage, analogous to refluxing in aqueous acetone in the presence of periodic acid (as a catalyst) would give a transdiaxial cholestan-3β, 5α,6β-triol which is another non-enzymic product of biological in vitro reactions. These autoxidation products were synthesized and purified for reference purposes.

Once an α-hydroxy group is inserted at the 7α-position then oxidation of the 3β-hydroxyl group seems to be the next step. From chemical considerations the specific oxidation of an equatorial less hindered hydroxyl can be achieved in preference to a more activated hydroxyl group at the 7α-position through an Oppenauer oxidation. In practice this reaction suffers from the ready dehydration of the 7α-hydroxy group to yield cholest-4,6-diene-3-one. Since the action of the 5-ene-3-keto isomerase during isomerization of the 5-ene-3-keto steroids to corresponding 4-ene-3-ketone can be compared with the acid- or base-catalysed reactions, the above-mentioned dehydration of the 7α-hydroxyl group would be expected to occur biologically unless the 7α-hydroxy group was protected. Chemically a temporary protection of the 7α-hydroxyl group as an ether is indicated (Greenhagh et.al 1952). Benzoylation and esterification do not protect the 7α-hydroxyl group from dehydrogenation under acid or alkaline conditions.

After the formation of cholest-4-en-3-one-7α-ol probably the next step is the 12α-hydroxylation. Discussing the

autoxidation reactions of paraffinic hydrocarbons Waters (1964) and Hawkins (1961) have mentioned that substances containing tertiary C-H are more easily oxidized to form hydroperoxides whilst methyl groups are more resistant. The methylene group in a straight chain saturated hydrocarbon or in cyclic hydrocarbon can be autoxidized similarly by molecular oxygen. High temperature and certain metals promote such reactions. It is therefore quite probable that hydroxylation at saturated centres like C-11,12,22,24 and 26 could proceed through intermediary hydroperoxide formation catalysed by enzymes, thereby avoiding the free radical type mechanism proposed for autoxidation, and perhaps reducing the energy requirements for the reaction.

The 7α-hydroxylations in this work were achieved through 7α-hydroperoxides of cholest-5-en-3β,26-diol, cholest-5-en-3β, 25-diol, cholest-5-en-3β-ol-24-one and cholest-5-en-3β,12α-diol, and the four corresponding triols were prepared.

Cholest-5-en-3β,7α,24ξ-triol, cholest-5-en-3β,7α,25-triol and cholest-5-en-3β,7α,26-triol can be randomly tritiated and injected into a bile fistula rat in order to confirm the notion that after any modification of the side chain 12α-hydroxylation cannot take place. Also their rate of conversion to chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) can also be studied in fortified mitochondrial preparations with a view to studying which one of these triols may be the principal intermediate.

It is generally believed that the conversion of the sterol 5α-series to the 5β-series or vice versa is possible by conversion to a 4,5 double bond or 5,6 double bond and hydrogenation. The catalytic hydrogenation of sterols with 5,6 double bonds gives almost exclusively the 5α-series if the molecule is devoid of a 3α-hydroxyl or other substituents. In the latter case solely compounds of the 5β-series are obtained. These results can be interpreted on the principle of steric hindrance during the catalytic hydrogenation, and arguments have been advanced that the 3α-substituents possess axial conformation in such compounds and because of their bulkier nature are able to compel preferential adsorption of the β-side rather than the normally more accessible α-side of the steroid molecule.

Conclusive evidence has been obtained that epimerization of the 3β-hydroxyl takes place through the 3-ketone formation and that the 5,6 double bond migrates to a position of conjugation with the carbonyl group before being reduced enzymically. Therefore, although chemically the reduction of the 5,6 double bond with a 3α-hydroxyl group in a sterol molecule affords exclusively the 5β-series, the same analogy cannot be drawn with the biological conversion of the 3β-hydroxyl group to the 3α-hydroxyl group in which case the 3-ketone formed would be reduced to give a 5-ene-3α-ol grouping followed by reduction of the 5,6 double bond to the 5β-series. Similarly on the basis of chemical evidence it is difficult to predict

that in a biological system the ketone will be reduced in preference to the 4,5 double bond to give a 3α-hydroxyl group. If such an enzymic reaction is compared with the metal hydride reduction of/an α,β-unsaturated ketone then the 3β-hydroxyl group (equatorial) would be expected to be formed because the 3-ketone is unhindered (Barton 1953). Catalytic reduction of 3-ketones in an acid medium is known to give axial hydroxyl groups (3α) but in the presence of a 4,5 double bond the saturation of the olefinic bond would take place first. Although chemical evidence also points that the catalytic reduction of cholest-4-en-3α-ol affords a mixture of/the 5α- and 5β-series both in neutral and acid environments the presence of a 7α-hydroxy group (axial) or a 12α-hydroxyl (axial) or both in such a molecule would be expected to increase the hindrance for the catalyst to approach from the α-side of the molecule, with the result that preferential β-attack would be directed and consequently reduction to the 5β-series would be achieved. However, no chemical analogy can be drawn in which case 3-ketones would give rise to 3α-hydroxyl groups in the presence of a 4,5 double bond. On the other hand if these enzymic reactions are compared with catalytic hydrogenations one would expect to reduce the 4,5 double bond first possibly exclusively to the 5β-series compounds, when α-hydroxyl groups at 7 and 12 are present, and the reaction would be favoured in an acid medium. The 3-ketone of the 5β-series would then give a 3α-hydroxyl (equatorial) in a neutral medium on catalytic

reduction. The same end can also be achieved by metal hydride reduction. Hence the action of the 5β-reductases can be compared with catalytic hydrogenation in acidic medium and the 3 -ketosteroid:(acceptor) Δ^4-oxidoreductase with that of the catalytic reduction in neutral solvents or metal hydride reduction.

In order to confirm the hindrance effect caused by the α-hydroxyl groups at the 7 and 12 positions, cholest-4-en-3-one, cholest-4-en-3-one-7α-ol and cholest-4-en-3-one-7α,12α-diol can be partially reduced catalytically under identical conditions and the ratio of the 5α-to 5β-compounds established. These compounds together with the 5β-cholestan-3-one-7α-ol and 5β-cholestan-3-one-7α,12α-diol have been synthesized for reference purpose.

OBJECT OF THE PRESENT STUDY.

This work was undertaken in order to synthesise chemical models of possible biological oxidation products of cholesterol. Various hydroxylated and oxygenated derivatives of cholesterol were synthesised, which could be used as reference compounds in the identification of intermediates encountered in *in vivo* studies of bile acid biosynthesis. With the methods available for random isotopic labelling of cold compounds these chemical models can also be used in biological studies both in *in vivo* and *in vitro* to test whether or not these labelled compounds were converted to the expected biological products, so that a conclusion could be drawn about the possibility of their being obligatory intermediates in the sequence of cholesterol catabolism to bile acids. Since sterols are notable for their ability to form molecular compounds and mixed crystals, this property often rendered purification by fractional crystallization almost impracticable or impossible. The application of modern methods of analysis revealed that in certain instances a compound synthesised by a published route gave physical and chemical characteristics comparable to those reported. Nevertheless by newer methods the material was shown to be heterogeneous. In all preparations chromatography was invariably used for the purpose of separation and the compound was regarded as pure when it gave only a single spot on TLC using several suitable solvent systems in which impurities if

any were detected. Optical rotations, ultra-violet and infra-red spectrascopy were employed as tools in determining the structure of the compound.

EXPERIMENTAL

EXPERIMENTAL

1. Melting points were determined on a Kofler micro-stage (microscope RCH) apparatus.

2. Optical rotations were measured on Hilger polarimeter M 412 in chloroform. Where the compound was not sufficiently soluble, minimum amounts of ethanol were employed.

3. Infrared spectra (IR) were recorded on an 'Infracord' spectrophotometer (Perkin-Elmer) using potassium bromide disc procedure. The IR figures numbers refer to the Infrared spectra given in the Appendix.II.

4. Ultraviolet absorption measurements were performed in an 'Optica' CF4DR double beam recording spectrophotometer and 'Unicam' S.P.500 spectrophotometer. The compound was read in an ethanol solution. For fluorescent thin layer chromatography an ultraviolet mercury lamp (Maximum emission 254 mμ) was used.

5. Radioactive measurements were performed in a Packard Tricarb scintillation system 314EX. The scintillation liquid consisted of 2,5-diphenyloxazole (PPO) 5 g. and 1,4-bis-2-(4 methyl-5-phenyloxazoyl) -benzene (POPOP), 0.03 g in toluene(1L).

Radioactive measurements were also performed on thin layer scanning device constructed in this department by Mr. A. Purdie.

Fig. 6

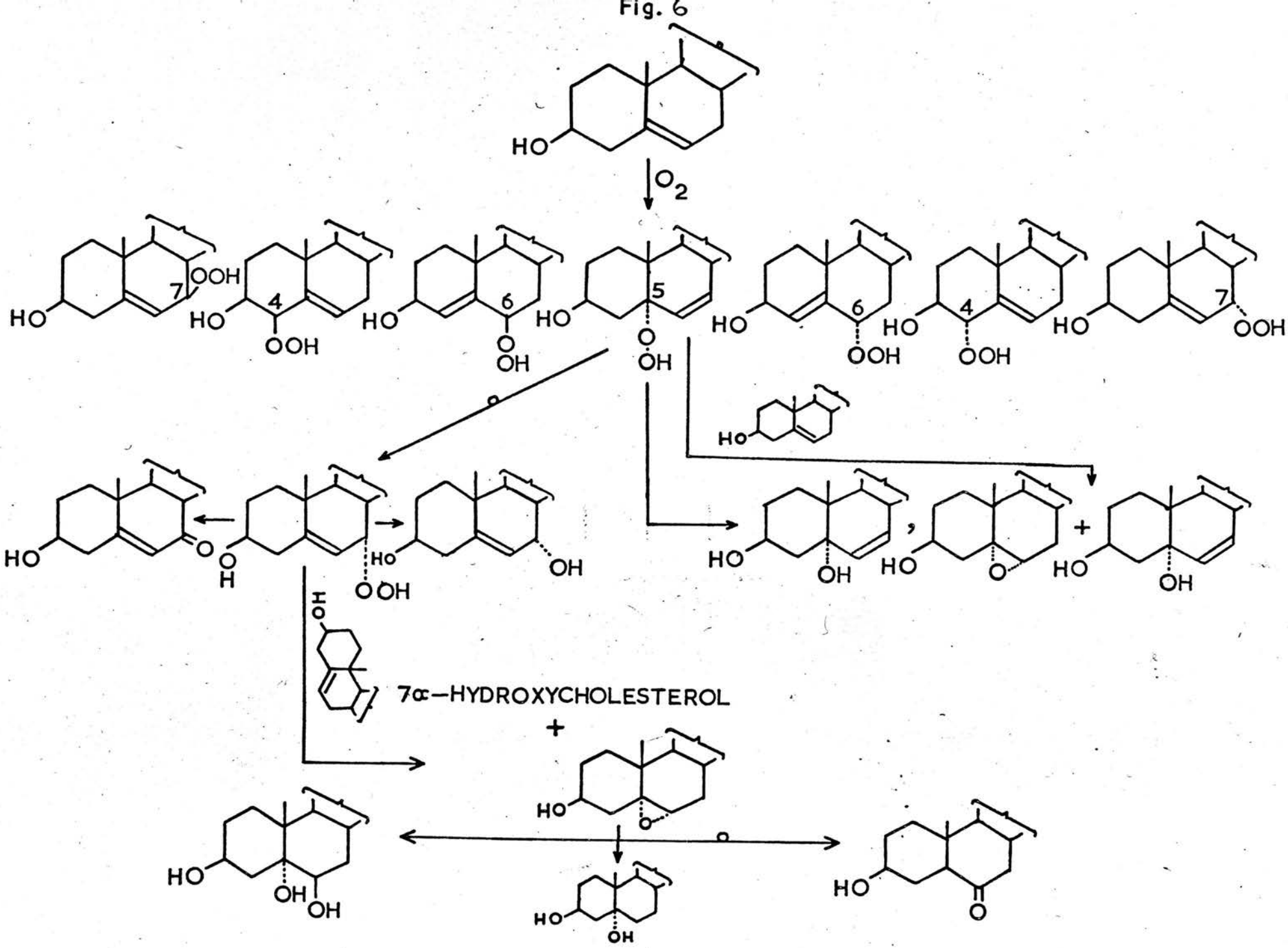


PHOTO-OXYGENATION

When molecular oxygen attacks a molecule such as cholesterol various hydroperoxy products are possible (fig. 6.). Under conditions that favour a free radical mechanism the attack of oxygen on allylic radicals can lead to both α- and β-hydroperoxides in almost equal proportions with rearranged and unarranged double bonds, because of the planar nature of the postulated free radicals. For example if the oxygen molecule is introduced at the 5-position cholest-6-en-3β-ol 5-α and 5β-hydroperoxides can result. If the attack is at the 6-position, both cholest-4-en-3β-ol, 6β-hydroperoxide and cholest-4-en-3βol-6α-hydroperoxide can be expected. A pseudo-allylic attack at the 4-position similarly may result in cholest-5-en-3β-ol-4β-hydroperoxide and cholest-5-en-3β-ol-4α-hydroperoxide. An allylic attack at the 7-position can form both cholest-5-en-3β-ol-7α-hydroperoxide and cholest-5-en-3β-ol-7β-hydroperoxide.

From these initial products other secondary compounds can be hypothesized. For example cholest-6-en-3β-ol-5α-hydroperoxide can isomerize to yield cholest-5-en-3β-ol-7α-hydroperoxide. It can react with one molecule of cholesterol to form cholest-6-en-3β,5α-diol and cholestan-3β-ol-5,6-epoxide, or it can be reduced to cholest-6-en-3β,5α-diol or cholestan-3β, 5α-diol. Similarly cholest-5-en-3β-ol-7α-hydroperoxide can form cholest-5-en-3β,7α-diol and cholestan-3β-ol-5,6-epoxide following its reaction with another cholesterol molecule.

It can also yield cholest-5-en-3β-ol-7-one on removal of a molecule of water, and it can also decompose to give cholest-5-en-3β,7α-diol and a ketone. On reduction cholest-5-en-3β-ol-7α-hydroperoxide can give cholest-5-en-3β, 7α-diol or 5α-cholestan-3β,7α-diol.

A corresponding series of compounds would be expected to be formed from cholest-4-en-3β-ol-6α-hydroperoxide, cholest-4-en-3β-ol-6β-hydroperoxide, cholest-5-en-3β-ol-4β-hydroperoxide and cholest-5-en-3β-ol-4α-hydroperoxide, but these compounds are not expected to undergo allylic rearrangement like the tertiary hydroperoxides to secondary hydroperoxides.

Further cholestan-3β-ol-5,6-epoxide can produce cholestan-3β,5α,6β-triol on hydrolytic cleavage of the oxide ring or it can rearrange to give cholestan-3β-ol-6-one. Reduction of cholestan-3β-ol-5α,6α-epoxide will afford mainly cholestan-3β,5α-diol, whereas cholestan-3β-ol-5β,6β-epoxide will give a mixture of cholestan-3β,5β-diol and cholestan-3β, 6β-diol.

Since this work was concerned with photo-sensitized oxygenations in dilute solutions (0.0025M) a different pathway seemed to have predominated in which hydroperoxidation was followed by an allylic shift of the double bond. Although this type of reaction predominated over the free radical pathway, nevertheless, the latter cannot be completely suppressed and therefore such products which are formed by the removal of/the allylic hydrogen may derive their origin from

the radical chain process particularly when α, and β-epimers at a certain position are formed.

MECHANISM:

The combination of molecular oxygen with olefins is well known and α-methylenic groups are potentially reactive centres, where the oxidation appears to be initiated by the removal of a hydrogen atom.

Under the influence of molecular oxygen, a labile methylene group can form a free radical to which oxygen forms a peroxyl radical. This molecule subsequently abstracts a hydrogen from a nearby labile site of another unsaturated molecule to form another free radical and thus propagates the chain reaction. Where several such methylene groups are present the most likely point of attack may be expected to be that one in which the tendency to dissociation of hydrogen will be most facilitated by normal electronic polarisation. This is roughly the mechanism advanced for the auto-oxidation of unsaturated fatty acids (Bolland and Koch 1945).

The lack of satisfactory source of energy required for the rupture of an α-methylenic carbon-hydrogen bond (80kcal/mole) led various workers to suggest an attack at the double bond as the initial reaction. This type of additive attack by oxygen need occur only to a minor degree at the double bond of a few molecules and then later continues as a substitutive attack at the allylic position during the predominating reaction. This again involves a free radical chain reaction in which a radical

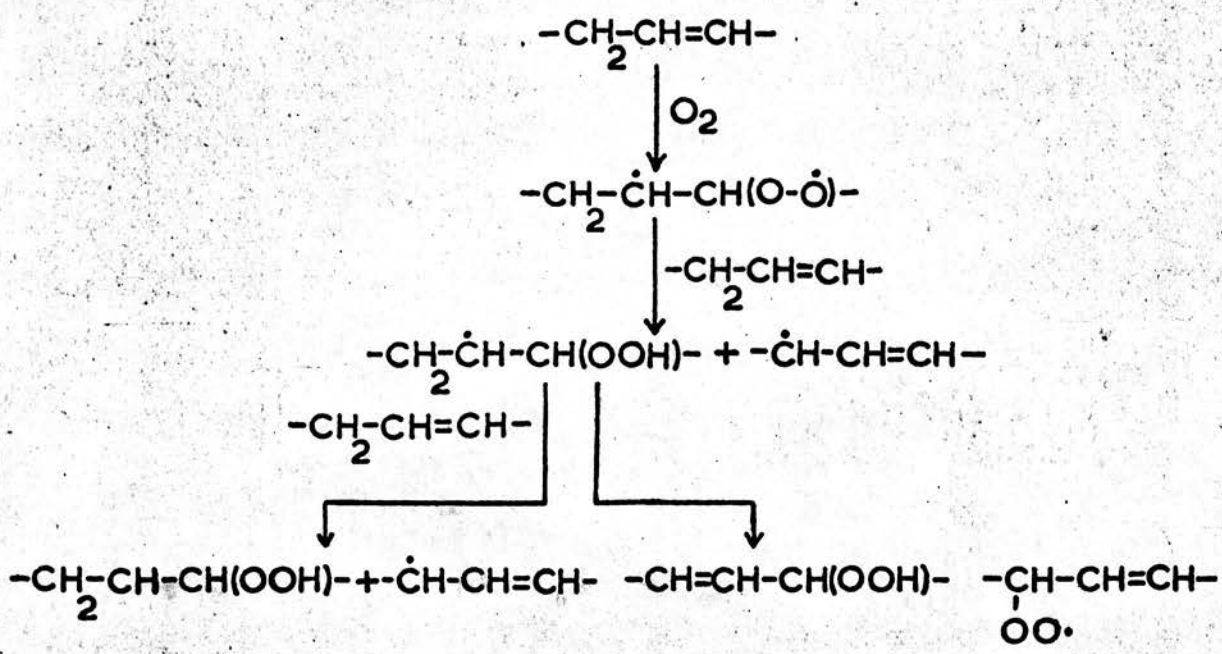

-CH2-CH=CH-
O2
-CH2-ĊH-CH(O-Ȯ)-
-CH2-CH=CH-
-CH2-ĊH-CH(OOH)- + -ĊH-CH=CH-
-CH2-CH=CH-
-CH2-CH-CH(OOH)- + -ĊH-CH=CH-
-CH=CH-CH(OOH)-
-CH-CH=CH-
OO·

fig. 7.

is formed with subsequent rearrangement and further reaction giving rise to saturated hydroperoxides as well. (fig.7.)

In contrast when oxygenation of sterols having the 5-ene-3β-ol grouping with or without one hydroxyl group elsewhere (12, 24, 25, 26 positions) in the molecule was conducted photochemically in dilute solutions in pyridine in presence of a small amount of sensitizing agent (haematoporphyrin) chain reactions of free radical type did not appear to be involved in any detectable extent and the mode of attack of oxygen was more stereospecific and markedly subject to steric hindrance.

The peroxides formed largely survived further breakdown. The initial and predominant mode of oxygen attack seemed to be at the C_5 of the 5-6 double bond from the rear of the sterol molecule giving rise to a biradical, which stabilized to an allylic hydroperoxide by allylic shift of the double bond, accompanied by hydrogen abstraction. The carbon-hydrogen bond cleaved and the carbon hydrogen bond laid down therefore required a cis relation, and the whole mechanism may or may not be concerted.

Studies with the ^{2}H-labelled cholesterol established that the α-hydrogen at C-7 is selectively abstracted and the result led to a postulation of a cyclic mechanism for the sensitized pathway (Nickon and Bagli 1959; 1961). Further work with other steroid olefins such as cholest-3-ene (Nickon et. al 1965) and cholest-4-ene showed that such attacks by a suitably

activated oxygen molecule on one of the termini of the double bond are on the π electrons from a direction perpendicular to the plane of the double bond. Such sensitizer mediated photo-oxygenations can be substantially blocked when the incipient C-O bond is in 1,3-diaxial relationship to an alkyl group or when the allylic hydrogen is rigidly equatorial or quasi-equatorial (Nickon and Mendelson 1965[a]).

The stereospecific behaviour of the 5-ene-steroids with regard to the mode of oxygen attack at the 5α-position can therefore be interpreted on the basis of a cis- mechanism proposed by Nickon and co-workers.

The attack at C-5 from the α-side of the sterol molecule by oxygen is greatly favoured because it meets no steric hinderance and the quasi-axial α-hydrogen at C-7 is available for the cis addition for production of a 5α-hydroperoxide. In contrast the approach of oxygen molecule from the β-side will encounter an almost eclipsed 1,2-interaction at C-5 from the C-10 methyl group. Further the allylic β-hydrogen at C-7 is quasi-equatorial, which is an unfavourable conformation for a cis-mechanism. These factors will therefore seriously discourage any frontal attack by molecular oxygen. Indeed no 7β-hydroxy isomer was found in the photo-oxygenation reaction mixture after isomerization in chloroform, reduction with $LiAlH_4$ and chromatography of the acetylated product on neutral, deactivated alumina. The absence of a 7β-hydroxy isomer explains three things. First there was no detectable amount

of 6-ene-5β-hydroperoxy derivative formed, since such a tertiary hydroperoxide would be expected to isomerize to a secondary 5-ene-7β-hydroperoxide derivative and thus would give a 7β-hydroxy derivative on reduction. This would be like cholest-6-en-5α-hydroperoxide isomerizing to cholest-5-en-7α-hydroperoxide, and the cholest-3-en-5α-hydroperoxide and cholest-3-en-5β-hydroperoxide isomerizing to cholest-4-en-3α-hydroperoxide and cholest-4-en-3β-hydroperoxide respectively. Secondly it explains that no decomposition to a 7-keto derivative occurs under our experimental conditions. Thirdly, it also explains that no 7-hydroperoxy derivative was formed through the free radical mechanism which would be expected to realize both α and β isomers.

The possibility of oxygen attack at the other termini of the 5,6 double bond i.e. C-6 is considered now. The α-attack of the oxygen molecule at C-6 meets no serious steric hinderance, but the 4α-hydrogen is equatorial, which is therefore unfavourably disposed for a cis mechanism. However, the ring A is free to assume different shapes ranging from/the thermodynamically most stable chair form of the cyclohexane ring to the less stable boat form. Therefore it is reasonable to assume that the 4α-hydrogen probably assumes an axial conformation as a result of ring A distortion, thus facilitating 6α-hydroperoxide formation. This reaction consequently will involve the shift of the double bond from the endo- to the exo-position with respect to the ring B.

Such a shift of the double bond may restrict this reaction but does not seem to completely prohibit it, by analogy with the formation of cholest-5-en-4β-hydroperoxide in 8.5% yield from cholest-4-ene among other major products of photochemical oxygenation.

The β-attack by molecular oxygen at C-6 is subject to a 1,3 interaction of the methyl group at C-10 with the developing C-O bond. The 4β-hydrogen (axial) although ideally situated for a cyclic transfer also experiences a 1,3 interaction with the methyl group at C-10. Therefore if the formation of a 6β-hydroperoxy derivative with an allylic shift of the double bond is on the pathway of photo-sensitized oxygenation reactions these interactions are insufficient to stop the reaction though they may retard it to a considerable extent.

As far as the proportion of 6α- and 6β-hydroperoxides is concerned, it will depend on whether the 1,3 interactions at C-4 and C-6 for the 6β-hydroperoxidation are more prohibitory than the ring A distortion for the 6α-hydroperoxidation.

The possibility of allylic oxidation by a free radical pathway cannot be completely eliminated, in photo-oxygenation in the presence of a sensitizer. Therefore it is worthwhile to discuss the possibility of oxygen attacking a 5-ene system through a free radical mechanism.

By analogy with the behaviour of other steroids this free

radical pathway is expected to produce a mixture of epimers at the point of attack of oxygen in the case of allylic free radicals, because in such a system the single p-electron (free) delocalizes in the direction of the adjacent 2 π electrons, as they are coplanar. For example a free radical type of oxygenation of/a colloidal solution of cholesterol produces both cholest-5-en-3β,7β-diol and cholest-5-en-3β, 7α-diol (Wintersteiner and Bergstrom 1941; Bergstrom and Wintersteiner 1941-42). Similarly photo-oxygenation of cholest-5-en-3-one in the absence of a sensitizer follows a free radical mechanism which produces approximately equal amounts of cholest-4-en-3-one-6α-hydroperoxide and cholest-4-en-3-one-6β-hydroperoxide (Nickon and Mendelson 1965b;Cox 1965). Autoxidation of steroid 3,5-diene-3-ol ethers similarly give 6α and 6β-hydroxy epimers (Gardi and Lusignani 1967). Other examples are given by Shapiro, Legatt and Oliveto (1964), for the hydroperoxidation of homo-allylic ketones to α,β-unsaturated ketones.

It has been found that no 5β-hydroperoxide or 7β-hydroperoxide were formed in the photochemical oxygenations hence it can safely be assumed that the 5α or 7α-hydroperoxide that were formed exclusively arose from the photo-sensitized pathway. Since both the 6α- and the 6β-hydroperoxides can be formed through a photosensitized path, it is very difficult to apply the same reasoning in order to exclude or include the chain reaction mechanism for their formation. The auto-

oxidative behaviour of cholesterol must be taken into account, recently Smith, Matthews, Price, Bachmann and Reynolds (1967) have studied autoxidation products under six different conditions. In all cases among a variety of products the only common feature was the presence of epimeric 7-hydroxycholesterols. These results demonstrate the outstanding reactivity of the cholesterol molecule at the allylic 7-position, as compared to other positions. Therefore it can be argued that since in photochemical oxygenations in the presence of a sensitizer no detectable free radical type of product was found at the 7-position, any hydroperoxy product formed at C-6 could not have originated through a free radical mechanism.

Similarly the formation of a free radical at C-4 seems remote and under photosensitized conditions no 4α or 4β-hydroperoxide arising by a free radical pathway seems possible.

EVIDENCE FOR OXYGEN ATTACK AT C-6

On catalytic reduction after isomerization of the photo-oxygenation products of cholesterol in pyridine a minor product cholest-4-en-3β,6β-diol was isolated and its identity established by co-chromatography on TLC and mixed melting point etc. This is therefore the first example of oxygen attack from the β-side of cholesterol molecule under the adverse 1,3-interaction of both the C-O forming and C-H breaking bonds.

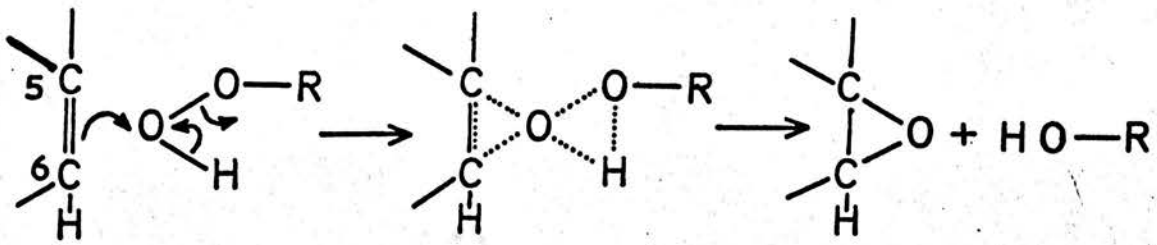
EVIDENCE FOR THE FORMATION OF 5,6-EPOXIDE

Even in the initial stages of the photo-oxygenation as

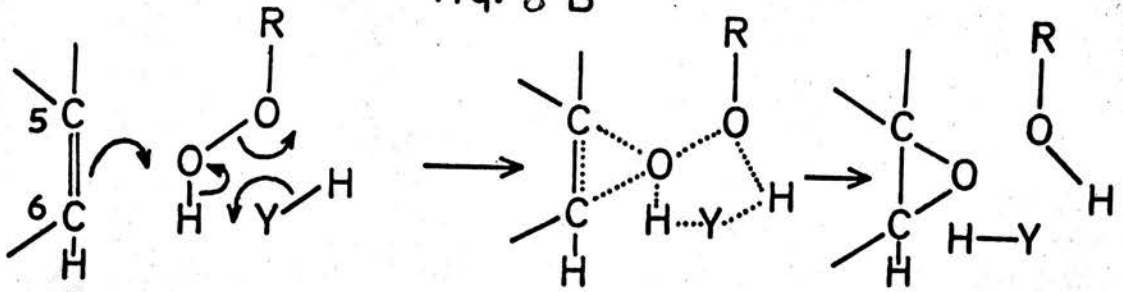
followed by fluorescent thin layer chromatography there were small spots with the mobilities of 5-ene-7α-hydroxy and 6-ene-5α-hydroxy products without any traces of 7-keto derivatives. This formation of hydroxy products from corresponding hydroperoxides in the absence of a ketonic product requires an explanation. The formation of a 7α-hydroxy product from a 7α-hydroperoxy product in pyridine solution is not surprising because the 6-ene-5α-hydroperoxide is known to decompose to 7-keto derivative in the presence of a sensitizer. This decomposition of a 6-ene-5α-hydroperoxide is known to involve isomerization to 5-ene-7α-hydroperoxide, which on dehydration would give a 7-ketone. (Schenck, Neumuller and Eisfeld 1958). Therefore it is possible that even in pyridine some of the 6-ene-5α-hydroperoxide is isomerized to the secondary 5-ene-7α-hydroperoxide. Indeed in the preparation of cholest-6-en-3β,5α-diol through photo-oxygenation of cholesterol in pyridine cholest-5-en-3β, 7α-diol was detected.

The absence of a ketone in the decomposition of 5-ene-7α-hydroperoxide or 6-ene-5α-hydroperoxide can be explained if a direct attack on the 5,6-double bond by these hydroperoxides to give simple 5,6-epoxides is proposed. This reaction will be analogous to the oxidation of olefins to epoxy-derivatives by peracids such as perbenzoic and perphthalic acids. However these hydroperoxides are expected to be less effective for this reaction than the peracids,

fiq. 8 A



fiq. 8 B



probably because there is no adjacent carbonyl group in these hydroperoxides, which would remove the peroxidic proton. The reaction might be initiated either by the polarisation of the π-electrons or by the loss of a peroxide proton, and might involve a chargeless transition state as given in fig.8A . Alternatively the transference of a proton might occur intramolecularly under the influence of a hydrogen bonding solvent (H-Y) as represented in fig.8B. Such a reaction therefore provides a possible explanation for the formation of 6-ene-5α-ol and 5-ene-7α-ol derivatives in the absence of any ketonic products in photochemical reaction mixtures. These epoxides (5α,6α and 5β,6β) are known to furnish trans-diaxial glycols on hydrolytic fission and monohydric alcohols on reductive opening. If a close analogy is drawn between photo-oxygenation and enzymic hydroxylations then the formation of cholestan-3β,5α,6β-triol and 5α-cholestan-3β-ol-6-one, the non-enzymic autoxidation products could well be explained through the formation of such epoxides, probably by an "enzymic" hydrolytic cleavage and isomerization respectively.

EXPERIMENTAL EVIDENCE FOR HYPOTHESISED MECHANISM

In a preliminary experiment cholesterol-4-C^{14} in benzene solution was added to the solution of cholest-6-en-3β-ol-5α-hydroperoxide in benzene and the mixture allowed to stand for five hours. An aliquot of the mixture was spotted in a line on the plate and then irrigated in benzene, ethyl acetate

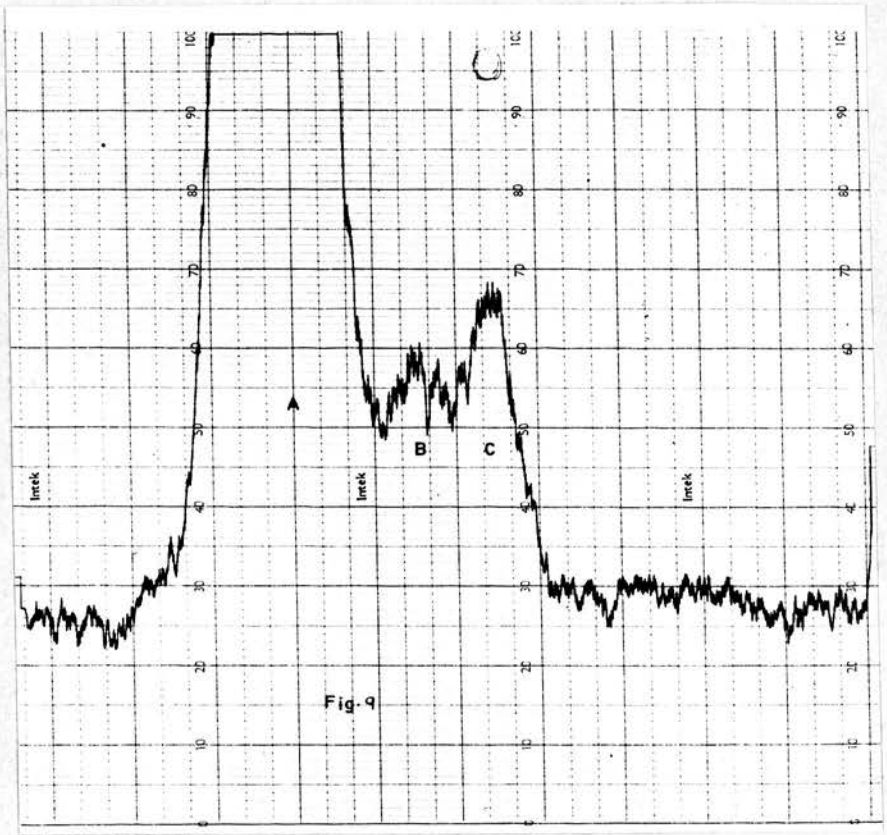


Fig. 9.

The radioactivity peak designated by'A'represents cholesterol.The peak'B' occupies an area covered by the reference compound cholest-6-en-3β-ol-5α-hydroperoxide, and the third peak designated by'C' has the same mobility as the reference compound 5α,6α-epoxycholesterol.

mixture (7:13). The TLC plate was cut into small sections, and the silicic acid scraped off into the counting vials and then radioactivity assayed in Packard Tri-carb Liquid Scintillation Spectrometer using toluene with PPO and POPOP. The main area of radioactivity was distributed in the region of cholestan-3β-ol-5α,6α-epoxide and cholestan-3β-ol-5β,6β-epoxide which do not separate from each other.

Conversion of the cholesterol-4-C^{14} to 5,6-epoxy-cholesterol-4-C^{14} was less than 2%.

The bulk of the reaction mixture was left at room temperature for several days and then chromatographed on TLC plate as described above. The plate was assayed for radio-activity using a windowless gas-flow TLC scanner. Three peaks of radioactivity corresponding to cholesterol, cholest-5-en-3β-ol-7α-hydroperoxide or cholest-6-en-3β-ol-5α-hydroperoxide and 5,6-epoxycholesterol were shown. (fig.9.)

The difficulty in the identification of such reaction products as 5,6-epoxides, 4-ene-6-isomeric hydroperoxides and probably the 5-ene-4-isomeric hydroperoxide (pseudo-allylic) is due to many factors:

1) They are not completely separated on TLC from each other or from the 6-ene-5α-hydroperoxides - the main product of the reaction of the 5-ene-7α-hydroperoxide.

2) Their isolation as such from the reaction mixture by chromatography or fractional crystallization is made difficult by their instability (except epoxides) coupled with

minute quantities.

REDUCTION WITH LITHIUM ALUMINIUM HYDRIDE

Several methods for the reduction of a hydroperoxide to corresponding alcohol have been reported. These include the use of NaI, $NaHSO_3$, $SnCl_2$, SO_2, $LiAlH_4$, Zn-acetic acid, H_2-platinum or palladium (Davies and Feld 1956; Davies 1958).

Hydride reduction was preferred over controlled catalytic reduction as the former reagent is considered to form complex anions from a simple metal hydride. These anions are nucleophilic reagents which normally attack polarised multiple bonds (e.g. >C=O, >C=N, -C≡N, -N=O) at the more positive atom, but usually leave the isolated carbon-carbon multiple bond unaffected.

SOLVENTS

In the case of sterols, photo-induced oxygenations have been conducted in many solvents. The photo-oxygenation of sterols having a 5-ene-3β-ol grouping with a suitably orientated hydrogen at the allylic carbon atom gives mainly two initially formed α,β-unsaturated hydroperoxides derivatives namely 6-ene-5α-hydroperoxide or 5-ene-7α-hydroperoxide depending upon the kind of solvent used; thus in chloroform or dioxane cholest-5-en-3β-ol, gave the 7α-hydroperoxy product, in contrast when pyridine, benzene, alcohol or acetone was used, hydroperoxidation occurred at the unsaturated tertiary carbon atom 5 with the subsequent shift of the double bond to the allylic 6,7-position. On this

basis these solvents can be classified roughly as chloroform-type or pyridine-type.

The suggestion that in chloroform solution, the attack of molecular oxygen is stereospecifically at the allylic 7∝-position (Schenck, Neumuller,& Eisfeld 1958) seems attractive but the source of energy required for the rupture of an ∝-methylenic carbon-hydrogen bond (80 kcal/mole) would be difficult to explain. If a free radical type of reaction is envisaged then both 7∝ and 7β-hydroperoxides will be expected unless 7∝-hydroperoxidation takes place by some abnormal mechanism. Further, since cholest-6-en-3β-ol-5∝-hydroperoxide is known to undergo mutarotation or allylic rearrangement which is catalysed by light in chloroform solution to form cholest-5-en-3β-ol-7∝-hydroperoxide (Schenck et. al 1958; 1958a; Lythgoe and Trippet 1959) it is reasonable to propose that the first and the main attack is at the tertiary terminal of the 5,6 double bond, and the 5∝-hydroperoxide formed through cis- addition then isomerises to a 7∝-hydroperoxide (for mechanism of isomerization see Schenck et. al 1958 and Lythgoe et. al loc.cit). The attack of oxygen at the C-6 with subsequent shift of double bond to the pseudo-allylic 4,5-position can not be overruled. In fact in the reduction product of photo-oxygenation performed in[a] chloroform solution of cholesterol a product less mobile than cholest-5-en-3β,7∝-diol has been detected having the same mobility as cholest-4-en-3β,6β-diol on TLC plate, apart from two main products which

corresponded to cholest-5-en-3β,7α-diol and cholest-6-en-3β, 5α-diol or cholest-5-en-3β,7β-diol, or a mixture of the latter two compounds. No attempt was made for further isolation or identification of the cholest-4-en-3β,6β-diol like product or characterization of cholest-6-en-3β,5α-diol or cholest-5-en-3β, 7β-diol at this stage.

Irrespective of the nature of mode of attack of oxygen for 7α-hydroperoxidation in chloroform solution although directly 7α-hydroperoxides could be obtained and on reduction these hydroperoxides would furnish the desired 7α-hydroxy derivatives. The method suffered from the drawback that the hydroperoxy compounds formed were very labile under the conditions of reaction and gave rise to both more mobile and less mobile break-down products. Even though the reaction mixture was stored at low temperatures after the removal of the photo-catalyst the break-down products were still formed. Further, during the reaction the break-down products were in some cases formed fast, hence the time of photo-reaction was limited and had to be stopped while there was substantial amounts of starting material unreacted. The instability of hydroperoxides could be due to the decomposition products of chloroform itself, which is known to give rise to acidic products like carbonyl chloride, hydrochloric acid and chlorine. No decomposition of a 6-ene-5α-hydroperoxide was detected in its isomerization to the more stable 5-ene-7α-hydroperoxide in chloroform (free from hydrogen chloride and alcohol) under

the influence of light and absence of air even after standing for several days at room temperature. On the other hand in pyridine solution the 6-ene-5α-hydroperoxy compound formed was stable and the reaction mixture could be stored at room temperature overnight without any decomposition. For this reason most of the reactions were conducted in pyridine and the photo-oxygenation continued until most of the starting material had been reacted.

SENSITIZERS (CATALYSTS)

A large number of synthetic and natural dye stuffs have been employed as photo-sensitizers in photochemical oxygenations for example certain dyes such as eosin, rose-bengal, or natural colouring matters like chlorophyll, carotenoids, anthocyanidins, haematoporphyrins, protoporphyrins etc. (Schenck et. al 1958a; Oster et. al 1959; Livingston and Owens 1956). The concentration of the sensitizer affects the rate of the reaction, and the quantum yield of the reaction decreases markedly with increasing dye concentrations (Oster et. al 1959). Some sensitizers act within five minutes of the light exposure while others have a lag phase of as much as twenty minutes.

Two mechanisms have been advanced. One mechanism implies that the sensitizer on absorption of light energy is excited to its singlet state (short lived, 10^{-9} sec.) with subsequent transition to a triplet state (metastable) which is a longer lived excited state. The metastable or phosphorescent state

(i) $Sens \longrightarrow {}^1Sens$

(ii) ${}^1Sens \longrightarrow {}^3Sens$

(iii) ${}^3Sens + {}^3O_2 \longrightarrow \cdot Sens\text{-}O\text{-}O\cdot$ (adduct)

(iv) $\cdot Sens\text{-}O\text{-}O\cdot + A \longrightarrow AO_2 + Sens$

(iiia) ${}^3Sens + {}^3O_2 \longrightarrow Sens + {}^1O_2$

(iva) ${}^1O_2 + A \longrightarrow AO_2$

A= acceptor ; Sens= sensitizer

fig. 10

interacts with triplet state oxygen molecules to form reactive labile adducts of sensitizer and oxygen. This intermediate then reacts with the acceptor olefin to form a peroxidic product. In the second mechanism the reactive intermediate is an excited singlet state of molecular oxygen (Foote and Wexler 1964; Corey and Taylor 1964; Kearn, Hollins, Khan, Chambers and Radlick 1967; Kearn, Hollins, Khan and Radlick 1967).

Both mechanisms can be represented as in fig.10.

In most cases pyridine was used as the solvent and haematoporphyrin was used as the sensitizer. In the case when chloroform was used, haematoporphyrin methyl ester was employed for solubility considerations.

LIGHT SOURCE

Various light sources had been used by Naqvi and Boyd (1964). Fluorescent lamps (Phillips TL AD, 15 w. 18" long), Tungsten filament lamps (8" long), Rubidium lamp (8"), and a HgZnCd lamp (Phillips, Holland: type 93145E) were tried and all gave the hydroperoxide. The fluorescent lamps were found to be the most satisfactory because with this lamp the temperature of the reaction mixture did not rise above 20°C, while with the other two kinds the rise in temperature was troublesome. In general an elevated reaction temperature produced more side products.

In all the experiments described in this work four fluorescent lamps were used for illumination purpose. The

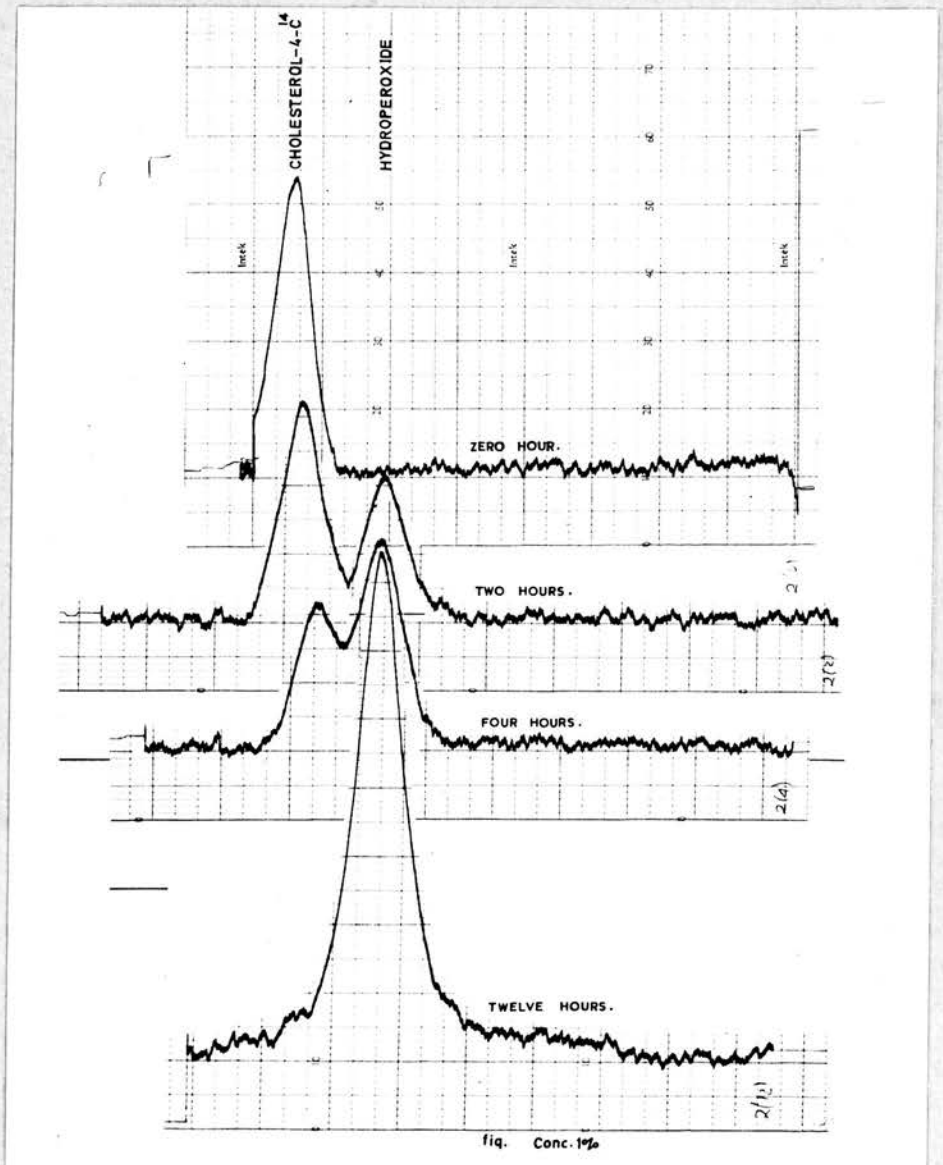


Figure. 11.

Thin layer radiochromatograms of the photo-oxygenation reaction mixture of cholesterol-4-C^{14} at different intervals, showing almost exclusive formation of a hydroperoxide.

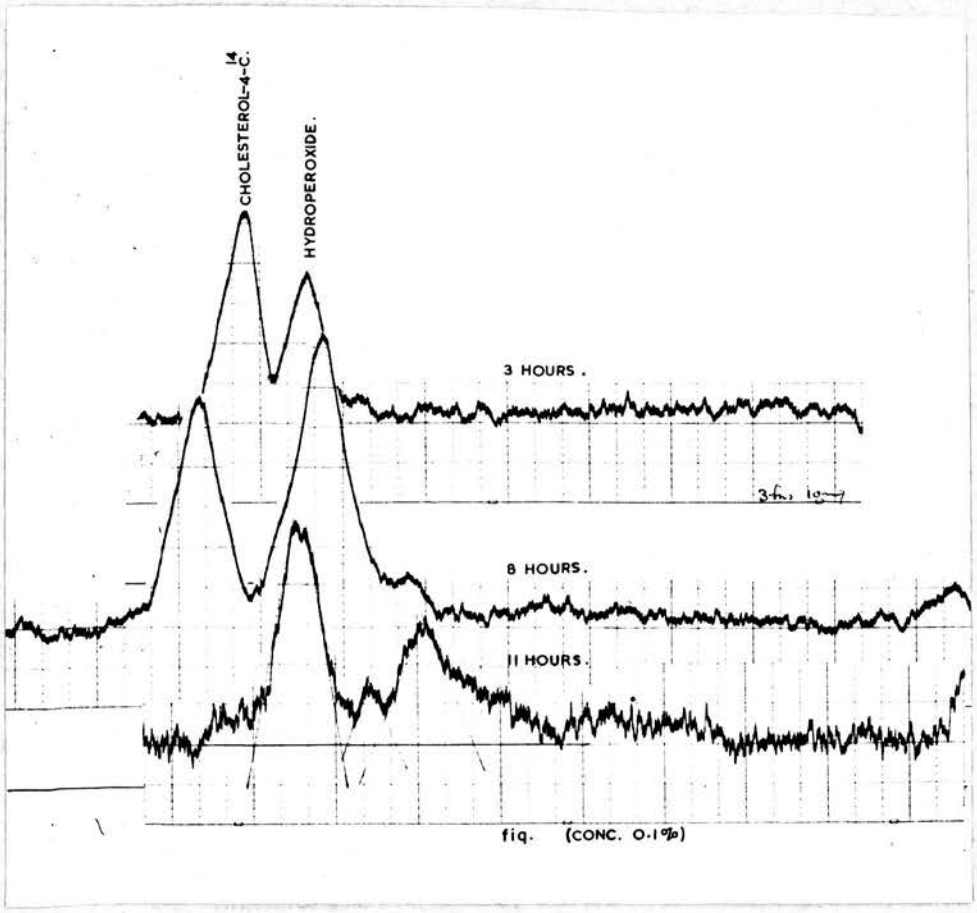


Fig. 12.

Thin layer chromatograms showing the formation of hydroperoxide and other pfoducts at different intervals of the photo-oxygenation of 4-^{14}C-cholesterol in pyridine.

reaction tube was made up of 'pyrex' glass provided with an oxygen inlet through a sintered disc at the bottom.

TIME COURSE

Cholesterol-4-C^{14} (1 μc) was diluted with 100 mg of cold cholesterol, and was dissolved in 10 ml pyridine containing 1 mg of haematoporphyrin. The solution was transferred into the broader limb of the U-shaped photo-oxygenation tube provided with a sintered glass at the bottom. Oxygen was led through the thinner limb via the sintered glass and the apparatus was illuminated by means of four fluorescent tubes (Phillips TL AD 15 w. 18" long). Aliquots (0.1 ml) were taken out after two hours, four hours and twelve hours, and were chromatographed on thin layer chromatoplates. Fig. 11, shows radio-chromatograms. After twelve hours of irradiation cholesterol was almost quantitatively converted to a product with the mobility of cholest-6-en-3β-ol-5α-hydroperoxide.

When the concentration of cholesterol was dropped to 0.1% or increased to 5%, the reaction did not seem to progress as smoothly as above (see fig.12).

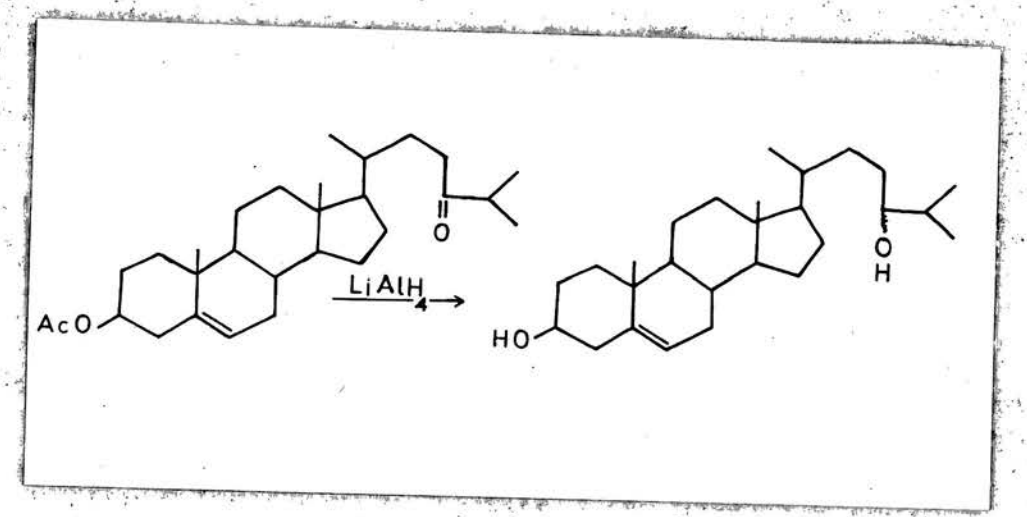


Figure 13.

Cholest-5-en-3β,24ξ-diol

3β-acetoxy-cholest-5-en-24-one (250 mg, IR=Figure 1. Rf.0.63 in benzene:petroleum ether:ether::2:2:1) was dissolved in freshly distilled tetrahydrofuran (THF) (6ml). The solution was carefully added to a suspension of lithium aluminium hydride ($LiAlH_4$) (150 mg) in THF (5 ml) and the mixture boiled under reflux for one hour. Ethyl acetate (10 ml) was added to decompose the excess hydride, and the whole poured into a saturated solution of potassium sodium tartrate (Rochelle salt). After standing for one hour the product was extracted with ether (4 x 15 ml). The ether extract was dried over anhydrous sodium sulphate and then evaporated to dryness in vacuo. The residue (0.242 g) was chromatographed on deactivated (5%) neutral alumina (10 g).

The reduction product was dissolved in benzene and passed through the column. Elution with increasing amounts of ethyl acetate in benzene furnished cholest-5-en-3β,24ξ-diol in 15% and 20% ethyl acetate fractions (20 ml each). These fractions were pooled and the residue (209 mg) was crystallized from ethyl acetate to give white needles (167 mg; Rf.0.53 in benzene:ethyl acetate:acetone::10:5:3).

	Reported		
m.p. = 169.5–172.5	isomer I = 176°	isomer II	183°
$[\alpha]_D^{26}$ = -29 (C,0.8)	-48		-26.8

IR (ν) = Fig. 2.

$C_{27}H_{46}O_2$ requires C = 80.5%, H = 11.4%; found C=80.69, H=11.64.

The low rotation of isomeric cholest-5-en-3β,24α-diol and cholest-5-en-3β,24β-diol mixture (for physical characteristic of these two isomers see Ercoli and De Ruggieri 1953) suggests that with $LiAlH_4$ reduction the former isomer predominates.

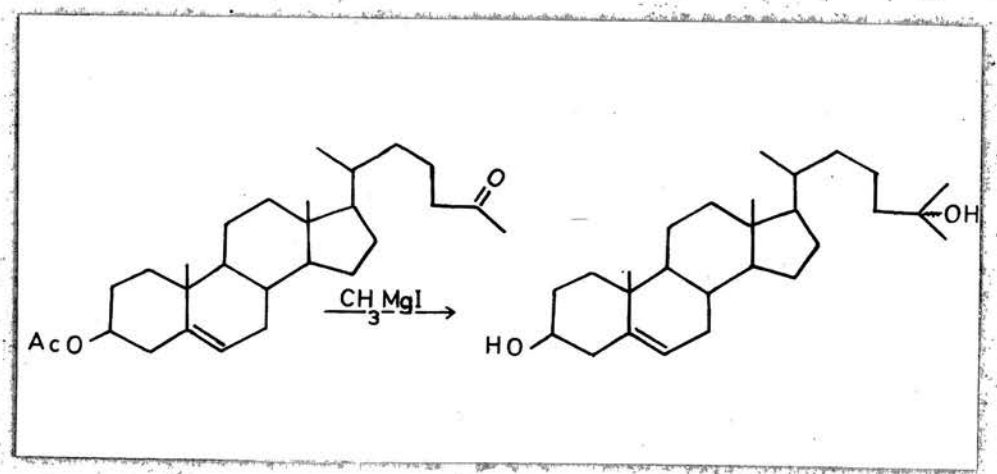


Figure 14.

Cholest-5-en-3β,25-diol (25-hydroxy-cholesterol)

25-hydroxycholesterol was prepared by the reaction of 3β-acetoxy-cholest-5-en-25-one (25 keto-nor-cholesterylacetate) with methyl magnesium iodide (Ryer, Gebert and Murrill 1950; Dauben and Bradlow 1950).

25-keto-nor-cholesteryl acetate was a gift from 'Organon', Newhouse, Lanarkshire. It showed several impurities on TLC and was purified by chromatography on deactivated (10%) neutral alumina.

Impure 25-keto-nor-acetate (5.9 g) was dissolved in the minimum volume of benzene and adsorbed on deactivated alumina (5g). The benzene was evaporated under vacuo and the sterol plus alumina poured on to a column of alumina (120g) made in petroleum ether. Elution with 10% benzene in petroleum ether (250 ml) removed the more mobile impurity which was identified as cholesterol acetate. Subsequently 20,30,40 and 50% benzene in petroleum ether (250 ml) gave pure 25-keto-nor-cholesterol acetate (4.6g m.p. 140-141.5 analytical sample crystallized from ethyl acetate, m.p. 142-144; IR=Figure 3. Rf. 0.46 benzene:petroleum ether:ether::2:2:1).

Further elution with 75% benzene followed by pure benzene gave a more polar product 'x' (150 mg m.p. 216-218.5°; Rf. 0.2) which will be described elsewhere.

A Grignard reagent was prepared from methyl iodide and magnesium in dry ether. Ether-washed and dried magnesium (0.96g; 40.0 m. moles) was dissolved in freshly distilled

methyl iodide (2.48 ml or 5.68 g; 40 m.mole) in anhydrous ether (50 ml). A solution of 25-keto-nor-cholesterol acetate (4 g; 9.3 m.mole) in dry benzene (25 ml) was added to the Grignard solution and the mixture refluxed for 4 hours and then left at room temperature for 12 hours. The product was decomposed with water (100 ml) followed by 50% acetic acid and finally steam distilled. The reaction mixture was extracted with ether, washed with water and dried over anhydrous sodium sulphate. The ether extract was evaporated and the residue dried at 80°C in a drying-pistol yielding 3.75 g of 25-hydroxycholesterol.

The analytical sample was prepared by chromatography of the foregoing material on deactivated (3%) neutral alumina (17 g). A suspension of 0.33 g in benzene (25 ml) was chromatographed. The material was applied to the column in 5% ethyl acetate in benzene (25 ml) and elution was achieved by 10%, 15%, 20% and 35% ethyl acetate in benzene. The 40-60% fractions were pooled and on evaporation gave 309 mg pure 25-hydroxycholesterol. Crystallization from methanol with small amounts of ether afforded 280 mg of white needles. Methanol alone furnished a white amorphous powder.

m.p. 182.5 Reported m.p. 182

$(\alpha)_D^{26}$ = -40 (C,0.5, d=0.10) -39

IR (ν) = Fig. 4.

$C_{27}H_{46}O_2$ requires C = 80.5%, H = 11.4%; found

C = 80.64 H = 11.45

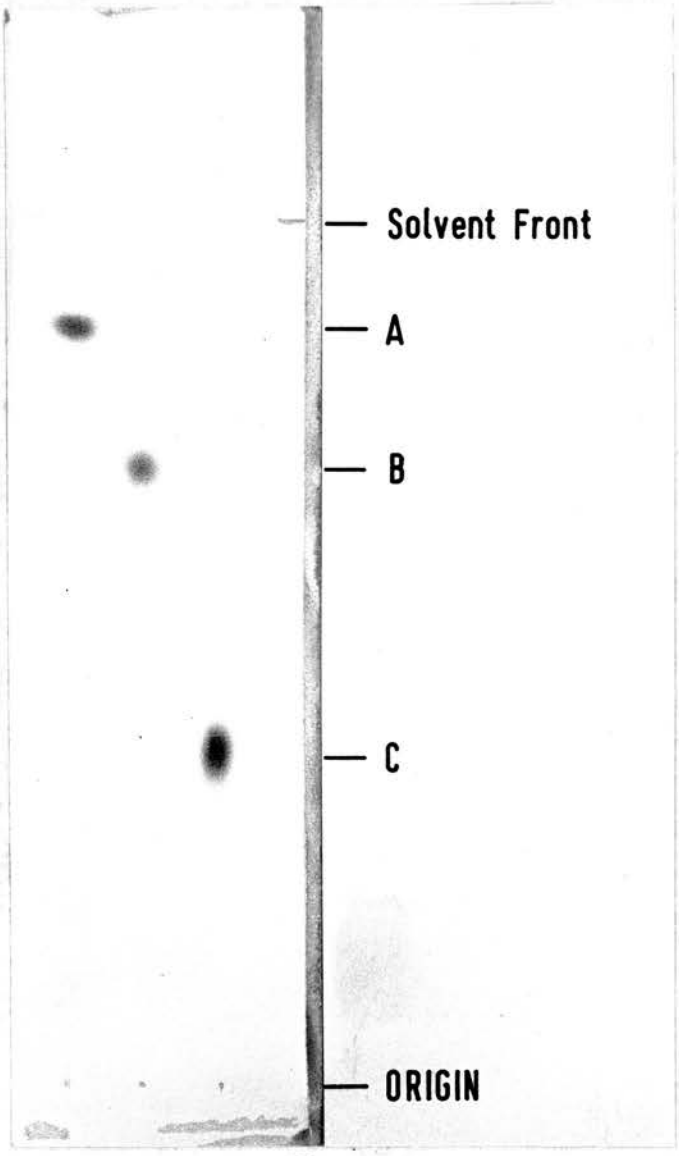


Fig. 15.

The alphabets A - C represent the following compounds.

Solvent system :- benzene : petroleum ether : ether :: 2 : 2 : 1.

(A) Cholest-5-en-3β-acetate.
(B) 25-keto-nor-cholesterol.
(C) 3β-acetoxy-20α-hydroxy-5-cholenic acid lactone.

Compound 'x':

Residue 216-218.5 from pooled fractions (see previous section) was dissolved in 40% benzene in petroleum ether and rechromatographed on deactivated (10%) neutral alumina (15 g).

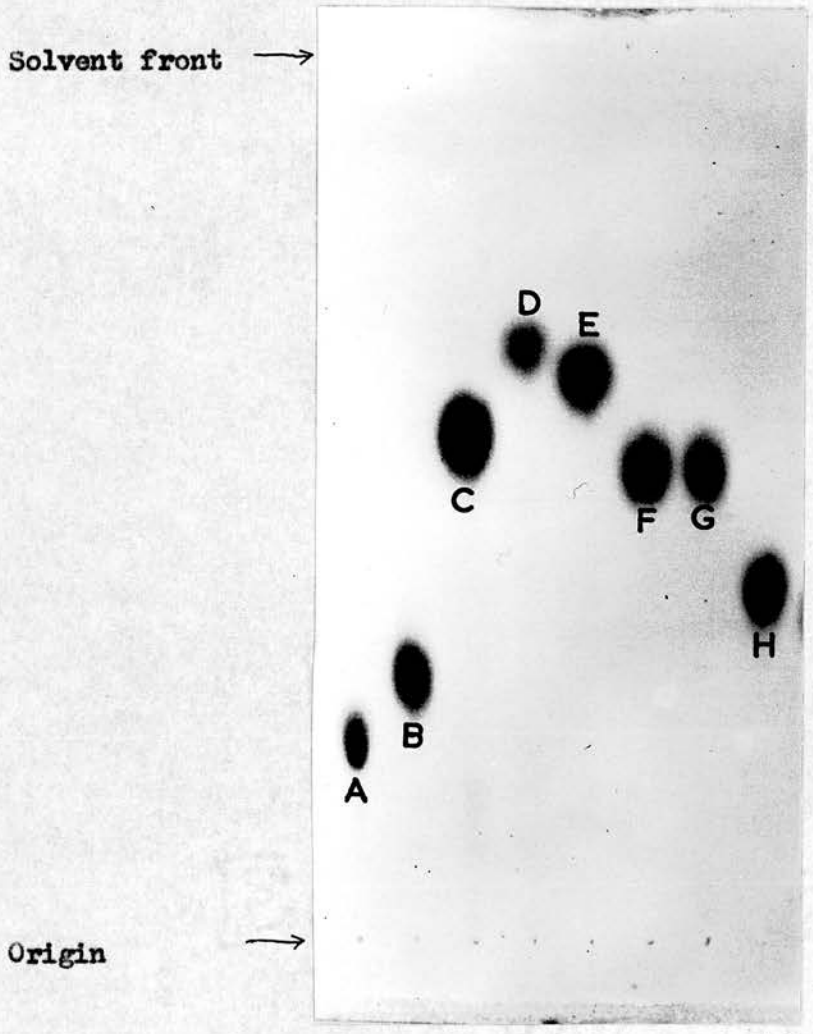
Elution was accomplished with increasing amounts of benzene followed by pure benzene and then 2% and 5% ethyl acetate in benzene. This gave 110 mg of a pure compound. In spite of chromatography the residue was slightly yellow, and was decolourised by treatment with activated charcoal in ethyl acetate solution. Crystallization of the residue from ethyl acetate - ether mixture afforded 90 mg of white needles, highly soluble in benzene and sparingly soluble in ethanol. The compound was more mobile than cholesterol on TLC (Rf. 0.75, benzene:ethyl acetate:acetone::10:5:3). Infrared spectrum revealed/the absence of hydroxyl peaks and presence of two ketonic peaks at 1738 cm^{-1} and 1763 cm^{-1}, the former can be ascribed to the carbonyl stretching of 3β-acetate, while the latter is typical of a γ-lactone (Ryer and Gebert 1952) carbonyl stretching vibrations. From these considerations and also that 25-keto-nor-cholesterol acetate was prepared by the chromic acid oxidation of cholesterol acetate dibromide, this compound 'x' was assigned the structure of 3β-acetoxy-20α-hydroxy-5-cholenic acid lactone.

m.p. = 222–223.5° Reported 218–219°

$(\alpha)_D^{26}$ = -25.0 (c,0.406;d,0.05)

IR (ν) = Fig. 5.

$C_{26}H_{38}O_4$ requires C=75.36, H=9.17; found C=75.74, H=9.4.



Thin layer chromatogram showing relative mobilities of the purified compounds in the solvent system, benzene : ethyle acetate :: 1 : 2. The alphabets A-H represent the following compounds.

(A) Cholest-4-en-3β,6β-diol.
(B) Cholest-5-en-3β,7α-diol.
(C) Cholest-5-en-3β,12α-diol.
(D) Cholest-5-en-3β,20α-diol.
(E) Cholest-5-en-3β,24ξ-diol.
(F) Cholest-5-en-3β,25-diol.
(G) Cholest-5-en-3β,26-diol.
(H) Cholest-5-en-3β,7β-diol.

Cholest-5-en-3β,26-diol

IR=Figure 6.

Kryptogenin (Cholest-5-en-3β,26-diol-16,22-dione) was purified by crystallization from ethyl acetate:MeOH, and 26-hydroxycholesterol was prepared according to the method of Scheer, Thompson, and Mossetig (1956).

1) Clemensen Reduction of Kryptogenin

Kryptogenin (5 g) was dissolved in ethanol (350 ml) and the solution was added to freshly prepared zinc-amalgam from 105 g zinc powder, 130 ml water, 10 g mercuric chloride and 6 ml conc.hydrochloric acid in an r.b. flask fitted with a condenser and a dropping funnel. The mixture was heated to reflux temperature and 84 ml of hydrochloric acid was added over 3 hours. Refluxing was continued for another 30 minutes. The reaction was followed on TLC (benzene:ethyl acetate: acetone::10:5:3). Kryptogenin was totally converted to more mobile products (3 spots) during the first twenty minutes, the most mobile being an unknown product, the least mobile was identified as cholest-5-en-3β,26-diol-16-one, and the middle spot being 26-hydroxy-cholesterol, which increased during the course of reaction, and the least mobile product decreased subsequently. At the end of three and a half hours there were three products more mobile than 26-hydroxy-cholesterol and two products less mobile than the 16-keto-diol.

The mixture was cooled, decanted off from zinc amalgam which was washed twice with 30 ml ethanol. Water was added to the ethanolic solution and extracted with ether. The

ether extract was worked up in the usual way. The residue was crystallized from hot benzene and the crystals were free from the more mobile impurities. Repeated crystallization of the needles obtained from benzene did not remove the traces of the keto-diol from 26-hydroxy-cholesterol. This keto-diol impurity could not be removed even after repeated treatment with Girard reagent T or P. In this way only a partial ketonic fraction was removed. Column chromatography on active (type '0' Peter Spence) as well as neutral alumina or silicic acid could not achieve their separation.

Finally the mixture of keto-diol and 26-hydroxycholesterol in methanol was reduced with an aqueous solution of sodium boro-hydride ($NaBH_4$) (1 g in 4 ml water) and worked up as usual. The reduction product was then chromatographed on deactivated (5%) neutral alumina.

The reduction product (3.0 g) was suspended in benzene and chromatographed on alumina (120 g). The column was eluted with benzene (250 ml), 0.3% MeOH in benzene, followed by 0.75% methanol in benzene (1100 ml). The last fraction gave pure 26-hydroxycholesterol which after crystallization from methanol gave 1.8 g of white needles.

m.p. = 177-178° Reported 177-178°

$(\alpha)_D^{27}$ = -31.9 -30

IR (ν) = Fig. 7.

$C_{27}H_{46}O_2$ requires C = 80.59, H = 11.44; found C = 80.61, H=11.28

2) By Huang-Minlon reduction

Kryptogenin (10 g) was reduced first with Clemmensen's method for two hours and forty-five minutes. The reaction mixture was worked up as before (total recovery 91%).

The recovered mixture (9.1 g) was heated with freshly distilled triethylene glycol (175 ml) and hydrazine hydrate (99-100%, 15 ml) on a heating mantle, in a r.b. flask fitted with an air condenser. After refluxing for half an hour, potassium hydroxide (17 g) was added carefully over a period of 20 minutes. Addition of potassium hydroxide depressed the boiling point of the mixture to 145°C. The air condenser was removed and the solvent was allowed to evaporate until the temperature reached 160°C, when the condenser was replaced and more hydrazine (5 ml) was added. The mixture was allowed to boil at 155-156° for half an hour. Finally the air condenser was removed and the temperature allowed to rise to 187°, and the mixture refluxed for two hours. The reaction mixture was allowed to cool and then poured into 0.5 N HCl (1000 ml) and left at 3°C for eighteen hours. White needles were collected on a sintered-glass funnel and then fractionally crystallized with ethyl acetate (4.3 g; 43%).

m.p. = 177-178.5°

$(\alpha)_D^{27}$ = 31.5

IR (ν) = Fig. 7.

One gram of slightly impure product was also obtained (m.p. 172-175).

Cholest-5-en-3β,26-diacetate

Mother liquors from the Clemmensen reduction and Huang-Minlon reaction were pooled (residue about 4 g) and acetylated at room temperature in pyridine and acetic anhydride. The acetylated product was poured on to crushed ice and extracted with ether, washed, dried and evaporated. The residue was chromatographed on silicic acid.

A solution of 20% benzene in petroleum ether was passed through the column, and elution was effected with increasing amounts of benzene followed by ether in benzene. In the 70%, 80% benzene in petroleum ether and 4% ether in benzene fractions about 0.7 g of pure diacetate was obtained which on crystallization from methanol afforded white needles (0.5 g; Rf.0.6 benzene:petroleum ether:ethyl acetate:: 40:20:5).

m.p. 128° reported 129°

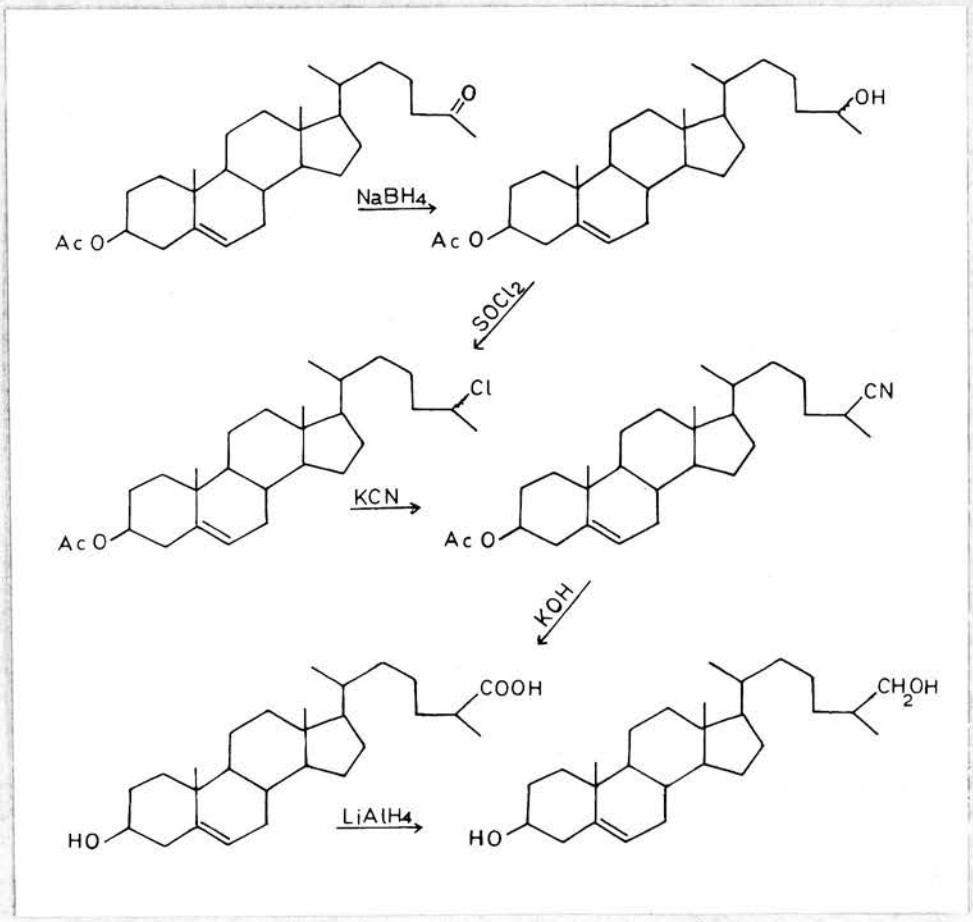

O
NaBH4
Ac O
OH
Ac O
SOCl2
Cl
Ac O
KCN
CN
Ac O
KOH
COOH
HO
LiAlH4
CH2OH
HO

Figure. 16.

Cholest-5-en-3β,25-nor-diol-3β-acetate

Sodium borohydride (0.3 g) was dissolved in aqueous methanol, and a solution of 25-keto-nor-cholesteryl acetate (0.4 g; Rf.0.73 in benzene:ethyl acetate:acetone::10:5:3) in ether (10 ml) was added slowly while stirring the solution gently. The reaction mixture was left at room temperature for one hour. Complete reduction to a more polar product was shown by TLC (Rf.0.72, solvent system same as above). The reduction product was worked up as usual, and the ethereal residue was crystallized from methanol to yield 25-hydroxy-nor-cholesteryl acetate as flat plates.

m.p. = 120° Reported 122°

$(\alpha)_D^{25}$ = -17.9

IR (ν) = Fig. 8.

$C_{28}H_{46}O_3$ requires C=78.1, H=10.7; found C=78.25, H=11.17

25-chloro-nor-cholesterol Acetate

25-hydroxy-nor-cholesterol acetate (Rf.0.33 in benzene: petroleum ether:ether::2:2:1.25) was dissolved in anhydrous ether (20 ml) and cooled to 0°, dry pyridine (0.1 ml) and freshly distilled thionyl chloride (0.1 ml) was added and the mixture left overnight. Approximately 50% conversion to a less polar compound was shown by TLC (Rf.0.82 in benzene: petroleum ether:ether::2:2:1.25). The mixture was refluxed for two hours, allowed to cool and then filtered. Pyridine hydrochloride was washed with more ether and the combined etheral solution was washed with $NaHCO_3$ solution (5%), water

and then dried over anhydrous sodium sulphate and evaporated. The residue (0.42 g) was dissolved in petroleum ether and chromatographed on neutral alumina (15 g). The column was eluted with increasing proportions of benzene in petroleum ether and finally 4% ethyl acetate in benzene gave pure 25-chloro-nor-cholesteryl acetate (0.27 g) on crystallization from petroleum ether as needles.

m.p. = 117.5°

$C_{28}H_{45}O_2Cl$ requires C=74.91, H=10.03, Cl=7.91; found C=75.15, H=10.07, Cl=7.70.

The column was further eluted with 20% and 40% ethyl acetate in benzene and 25-hydroxy-nor-cholesteryl acetate (120 mg) was recovered.

25-cyano-nor-cholesteryl-acetate

0.25 g, 25-chloro-nor-cholesteryl acetate (Rf.0.68 in benzene:petroleum ether:ether::2:2:1) was dissolved in dimethyl sulphoxide (5 ml) and refluxed with KCN (0.25 g) under nitrogen. The reaction was followed on TLC. No further reaction took place after four hours. The reaction mixture was cooled, poured into water and extracted with ether. The ethereal extract was washed free of cyanide ions with water (no ppt. with $AgNO_3$ solution) dried and evaporated. The residue showed one main spot corresponding to 25-cyano-nor-cholesteryl acetate (Rf.0.57) and three smaller spots corresponding to the starting material, 25-chloro-nor-cholesterol (Rf.0.185) and 25-cyano-nor-cholesterol (Rf.0.13).

Cholest-5-en-3β-ol-26-oic Acid

The above mixture was not further purified but was hydrolysed in aqueous-dioxane (20%, 5 ml) and KOH (150 mg) at reflux temperature for 8 hours, under nitrogen, cooled, diluted with water and extracted with ether (2 x 5 ml) to remove non-acidic material. The aqueous phase was acidified with dilute hydrochloric acid and left overnight, filtration gave an insoluble precipitate, which was washed neutral with water and dried. Recrystallization from ethanol and water gave cholest-5-en-3β-ol-26-oic acid (Ca.100 mg) (m.p. 172-175° reported 176-178°).

When hydrolysis was performed in alcohol for 30 hours, almost all of the 25-cyano-nor-cholesterol was recovered from the alcoholic hydrolysate after dilution with water and extraction with ether. Very little acidic product could be extracted with ether after acidification.

Cholest-5-en-3β,26-diol

Cholest-5-en-3β-ol-26-oic acid (50 mg) was dissolved in tetrahydrofuran (2 ml) and was dropped in a suspension of $LiAlH_4$ (100 mg) in THF (5 ml). The mixture was refluxed for two hours and worked up as usual. Chromatography of the product on neutral alumina furnished pure cholest-5-en-3β,26-diol in 20% and 30% ethyl acetate in benzene. The residue from these fractions was crystallized from benzene and white needles of cholest-5-en-3β,26-diol were obtained. The

compound was identical with that obtained from kryptogenin.

Melting and mixed melting point = 177°- 179°.

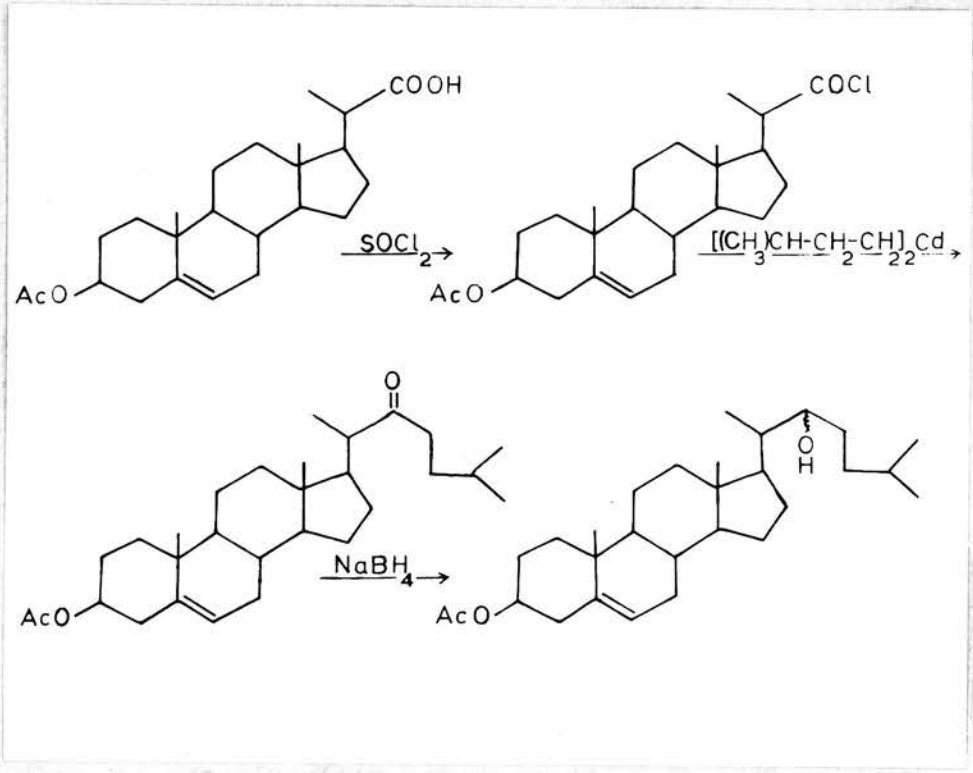


Figure. 17.

Cholest-5-en-3β,22ξ-diol-3β-acetate

3β-Hydroxy-bisnorchol-5-enic acid (5 g) was refluxed with acetic anhydride (3 ml) in a mixture of pyridine and benzene. Recrystallization of the residue from acetone/benzene afforded 3β-acetoxy-bisnorchol-5-enic acid (3 g) which was dissolved in freshly distilled thionylchloride (10 ml) and left overnight. Dry benzene was added, taken to dryness in vacuo and the residue crystallized from a small volume of benzene, (yield 1.0 g). The acid chloride was then thoroughly dried under high vacuum.

A Grignard reagent was prepared from 0.23 g of magnesium in ether (40 ml) and 1.51 g of iso-amyl bromide, and refluxed for a further fifteen minutes. The flask was cooled in an ice-bath and anhydrous cadmium chloride powder (0.9 g) was added while stirring. The ice-bath was removed and the mixture refluxed for 45 minutes. To the suspension of liquid di-isoamylcadmium in ether was added a benzene solution of the acetoxy-bisnorcholenic acid chloride, and the mixture refluxed for one hour, and left overnight at room temperature. Hydrolysis was accomplished with 10% hydrochloric acid, the ether layer was separated and the acidic layer re-extracted thrice with ether. The ether extracts were combined, washed until neutral with water, dried over sodium sulphate and evaporated to dryness. The residue was chromatographed on deactivated (5%) neutral alumina (100 g). The column was prepared in petroleum ether and the residue dissolved in 20%

benzene in petroleum ether was adsorbed. Elution with 40%, 60% and 75% benzene in petroleum ether afforded cholest-5-en-3β-acetoxy-22-one on evaporation. The residue was crystallized from ether-methanol and pure cholest-5-en-3β-acetoxy-22-one was obtained (Rf.0.59 in benzene:petroleum ether:ether::2:2:1).

m.p. = 152-153° Reported 154-155

$(\alpha)_D^{26}$ = -63.3 (c,0.505;d,0.16) -63.7

IR (ν) = Fig. 9.

$C_{29}H_{46}O_3$ requires C=78.73, H=10.40; found C=78.71, H=10.15

The mother liquor was evaporated and the residue (300 mg) was reduced with Na BH_4 in aqueous methanol in the usual way. The reaction mixture was left overnight at room temperature. TLC assessment on the following morning showed incomplete reduction, therefore it was refluxed for one hour. The reduction mixture was acidified and extracted with ether, washed until neutral, dried and evaporated. The residue (320 mg) was chromatographed on neutral alumina (15 g) deactivated with 1% water.

CHROMATOGRAPHY

The reduction mixture was dissolved in petroleum ether and passed through the column. Elution of the column with 20%, 50%, 80% benzene in petroleum ether and then pure benzene left no residue on evaporation. Ethyl acetate in benzene (2%, 5% and then 10%) removed some more mobile impurity and 15% and then 20% ethyl acetate in benzene

fractions (25 ml each) gave pure cholest-5-en-3β-acetoxy-22ξ-ol (Rf. 0.63 in benzene : ethyl acetate :: 1:1).

m.p.$_{27}$ = 168-169.5°

$[\alpha]_D$ = -68.0 (c, 0.31; d, 0.105).

IR (ν) - Fig. 10.

$C_{29}H_{48}O_3$ requires C=78.37, H=10.81; found C=78.48, H=10.67

Cholest-5-en-3β,4β-diol

This compound was prepared according to the method of Rosenheim and Starling (1937).

Selenium dioxide (2.5 g) was dissolved in water (1.5) and glacial acetic acid (50 ml) was added. The solution was heated to 80°C, and was rapidly added to the benzene (25 ml) solution of cholesterol (5 g) also heated to 80°C. The mixture turned yellow and finally red. After one hour refluxing, sodium acetate (10 g) was added and the heating continued for an additional 10 minutes. The solution was filtered into half-saturated sodium chloride solution (100 ml) and the benzene layer separated, washed with water, dried over sodium sulphate and evaporated under vacuum. The residue was washed with petroleum ether, and crystallized once with acetone and finally with 85% methanol, to yield slightly yellowish needles.

m.p. = 176° Reported 176-177°

$(\alpha)_D^{26}$ = -61.0 (c,1.0;d,0.305) -60

IR (ν) = Fig. 11.

$C_{27}H_{46}O_2$ requires C=80.59, H=11.44; found C=80.79, H=11.83

Cholest-5-en-3β,7α-diol

Photo-Oxygenation of cholesterol.

Cholesterol (5.0 g) and haematoporphyrin (14 mg) were dissolved in dry pyridine (100 ml) and photo-oxygenated for fourteen hours. The reaction was followed on TLC as described in details elsewhere. After six hours 50 per cent conversion was observed on the TLC plate (cholesterol Rf.0.76; 5α-hydroperoxy product Rf.0.5 in benzene:ethyl acetate::3:2)

Pyridine was removed under high vacuum on a water-bath not exceeding 45° using solid CO_2 in acetone traps. The last traces of pyridine were removed by treatment with dry chloroform free from alcohol, and subsequent evaporation until a dry residue was obtained.

The residue was treated with dry chloroform (100 ml) left overnight in the dark and filtered the following morning. The residue (R_1) was resuspended in sufficient dry chloroform and left for 48 hours for isomerization with occasional shaking at room temperature. Some of the residue did not dissolve during the first twelve hours and more chloroform was added. After forty-eight hours the solution was filtered to remove any suspended material and the filtrate evaporated under vacuum on a water-bath maintained at 45°C. The residue (2.0 g) was dissolved in ether (1000 ml) and reduced with hydrogen using palladium-on-charcoal catalyst (0.5 g). After one hour no spot corresponding to hydroperoxide was detected on TLC plate and the catalyst was filtered and the ether

evaporated. There were two spots on TLC plate, the most polar corresponding to the 7α-hydroxy-cholesterol, and the second was an unknown product having the same mobility as cholesterol. The ether residue was dissolved in 30% ether in petroleum ether (60-80) and chromatographed on silicic acid (50 g). The column was eluted with increasing amounts of ether in petroleum ether and 100 ml fractions were collected. Ether (90%) in petroleum ether (200 ml) gave pure 7α-hydroxy-cholesterol (1.6 g). Crystallization from methanol afforded 1.3 g of white needles.

m.p. = 187-188° Reported 188°

$(\alpha)_D^{22}$ = -91.4

IR (ν) = Fig. 12.

$C_{27}H_{46}O_2$ requires C=80.59, H=11.44; found C=80.83, H=11.6

The filtrate from residue (R_1) was treated with charcoal to remove the catalyst. Filtration and then evaporation gave a residue which was dissolved in 20% ether in petroleum ether and chromatographed on silicic acid (3% water) and 30% and 35% ether in petroleum ether eluted cholesterol, while the 45% ether fraction gave a residue m.p. 152°-155°, $(\alpha)_D^{22}$ - 120 which showed a single spot on TLC. Catalytic reduction of this residue gave several spots on TLC. From the reduction mixture pure 7α-hydroxycholesterol could not be obtained by direct chromatography. Chromatography of the acetylated mixture was not attempted.

In another experiment when photo-oxygenation was conducted

for twenty-three hours the overall yield of 7α-hydroxy-cholesterol was only 18%.

When a portion of the filtrate obtained from the R_1 residue above was reduced catalytically (H_2-Pd on C) white needles were deposited during the reduction. The reaction mixture was filtered and the crystals mixed with catalyst were thoroughly washed with a generous amount of ether. The residue was then extracted with tetrahydrofuran and evaporation of this solvent gave a powdery residue which was sparingly soluble in hot alcohol. The amorphous powder was crystallized from pyridine-methanol in white needles. The identity of this compound was established by mixed melting point with known synthetic cholest-4-en-3β, 6β-diol (page) and co-chromatography.

m.p. = 257-260°

Chromatographic separation of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol through their diacetates.

A mixture of cholest-5-en-3β,7β-diol and cholest-5-en-3β,7α-diol was obtained by the reduction of cholest-5-en-3β-ol-7-one by means of $NaBH_4$. The reaction product was acetylated in pyridine at room temperature for 12 hours and worked up as usual. The mixture of diacetates thus obtained did not separate on TLC and run as a single spot (Rf.0.52 in benzene: petroleum ether:ether::2:2:1; cholesterol acetate and cholesterol had Rfs.0.84 and 0.22 respectively). The mixture (600 mg) was chromatographed on neutral alumina (50 g). A solution of diacetates in petroleum ether was passed through the column and eluted with 20%, 40%, 60%, 80% benzene in petroleum ether followed by 2% ethyl acetate in benzene. These fractions (100 ml each) gave more mobile impurities. The later 4% ethyl acetate in benzene fraction (100 ml) gave a residue (340 mg) from which a few mg on hydrolysis with 5% methanolic NaOH gave two spots on TLC corresponding to the 7β- and 7α-hydroxycholesterols (Rf.0.25 and 0.2 respectively in benzene:ethyl acetate::1:1). Then 6% ethyl acetate in benzene gave a residue (130 mg) which on hydrolysis afforded pure cholest-5-en-3β,7β-diol. Further elution with 10% and 20% ethyl acetate in benzene followed by elution with pure ethyl acetate gave partially hydrolysed products (31 mg).

Physical characteristics of cholest-5-en-3β,7β-diol

m.p. = 178° Reported 178°

$[\alpha]_D^{26}$ = +15 +7

IR (ν) = Fig. 13.

$C_{27}H_{46}O_2$ requires C=80.5, H=11.4; found C=79.98, H=11.79

Residue from the 4% fraction (340 mg) was again chromatographed on neutral alumina (35 g) and eluted as described above using 50 ml eluants each time. Up to 2% ethyl acetate in benzene left no residue on evaporation. However the 3% ethyl acetate in benzene was collected in two portions - 30 ml and 20 ml. The residue from the 30 ml portion was hydrolysed and pure cholest-5-en-3β,7α-diol (10 mg) was obtained. The 20 ml portion gave a residue (76 mg) which on hydrolysis furnished a 1:1 mixture of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol. The 6% and 8% ethyl acetate in benzene fractions gave a residue (176 mg) which on hydrolysis gave pure cholest-5-en-3β,7β-diol. Later the 50% ethyl acetate in benzene gave a partially hydrolysed product (36 mg) which on hydrolysis gave a mixture of isomeric diols in almost equal proportions.

Physical characteristics of (a) cholest-5-en-3β,7β-diol

m.p. = 178°

(b) cholest-5-en-3β,7α-diol

m.p. = 187-188°

Cholest-5-en-3β-ol-7-one (7-ketocholesterol)

Cholest-5-en-3β-acetoxy-7-one was prepared according to the method of Fieser, Fieser and Chakravarti (1949) using chromic anhydride in glacial acetic acid and cholesterol acetate. 7-ketocholesterol acetate (3.9 g) thus obtained IR=Figure 14. was hydrolysed in methanol (500 ml) and potassium carbonate (3 g in 40 ml water) at room temperature for twenty-four hours. The solution was warmed, diluted with water and left for crystallization. Crystals were separated, washed, dried, dissolved in 10% ether in petroleum ether and chromatographed on neutral alumina (80 g). Ether eluted cholesterol and 7-ketocholesterol was obtained by elution with 10% acetone in ether followed by 50% acetone in ether (m.p. 169-171°).

Separation of 7α and 7β-hydroxycholesterols

A mixture of 7α- and 7β-hydroxycholesterols was obtained by the reduction of 7-ketocholesterol with sodium borohydride. For the separation of these two isomeric diols, the mixture was placed on a neutral alumina column with varying amounts of water. The column was prepared in petroleum ether and eluted with 10% acetone in petroleum ether. The solid mixture was stirred occasionally to facilitate its solution; care was taken not to disturb the alumina surface. In all cases 15% acetone in petroleum ether invariably dissolved all the solid mixture on the column. More alumina of the same activity was then added to the column to give a 1 cm. covering layer. The results are given in tabular form. The percentage of

water on the alumina was critical for a good separation of these isomers. When active neutral alumina was used or when deactivated (3% water) alumina was used almost no separation of the two isomers could be achieved. Increasing the activation of the alumina from 3% to 2% water improved the resolution of the two isomers and the best separation was achieved when 1% water was used for deactivation purposes.

Physical characterestics of (a) cholest-5-en-3β,7β-diol

m.p.	=	178°	Reported 178°
$[\alpha]_D^{26}$	=	+15	+7

(b) cholest-5-en-3β,7α-diol

m.p.	=	187-188°	Reported 188°
$[\alpha]_D^{26}$	=	-94.9	-90

% Water	Alumina wt.in gms.	Sterol mixt. in gms.	Fract. in mls.	7β in mgs.	Mixture of 7β and 7∝ in mgs. Parentheses shows approx. ratio of β:∝	7∝ in mgs.	%Acet. in pet. ether	Total recovery in gm.
3	30	0.39						
			40	20		trace	27.5	
			25		180 (2:1)		30	
			"		105 (1:1)		32	.381
			"	trace		66	15%alc	
			"			10	25%EtoH	
2	20	.15						
			25	2-3			30	
			"	26-27			32	
					58 (8:1)		34	.148
					43 (1:2)		36	
						16-17	40	
2	30	.24						
			40	3-4			28	
			"	20			30	
			4	7			32.5	0.23
			36		76 (8:1)		"	
			4		15 (1:1)		35	
			4		14 (5:4)		"	
			32	trace		33	"	
			40	"		34	40	
1.52	25	0.15						
			25	1			34	
			"	2			36	
			10	13			38	
			15	23-24			"	.143
			25		30 (4:1)		40	
			"		43 (1:9)		46	
						32	acetone	
1	25	0.175						
				trace			42	
				20			46	
				50			50	.180
					50 (5:4)		54	
				trace		45	60	
						15	65	
0	20	0.11						
			25	trace			60	
				1-2		trace	65	
			4		3 (8:1)		70	
			21		29 (1:1)			
					50 (1:2.5)		75	.103
					20 (1:8)		80	
						trace	84	

5β-Cholestan-3α-12α-diol (Dihydroxy coprostane-DHC)

Anodic Coupling of Deoxycholic and Isovaleric Acids:

To a solution of Deoxycholic Acid (m.p. 174-176°, 20 g; 0.0685 molar) in methanol (700 ml) was added a solution of sodium methoxide (3 g sodium metal dissolved in 100 ml methanol) and Isovaleric acid (87 ml or 81 g; molar ratio 1:12). The solution was electrolyzed between two Pt-plates (1" x 2") as electrodes, 0.2 cm. apart for nineteen hours with 18 v. and 1.75 amps in a beaker cooled with surrounding icewater. The reaction was followed on TLC plates every three hours (solv.system chloroform:acetone:ethanol:acetic acid:: 54:19:7:0.3 ml) and at the end of eighteen hours there was a trace of starting material (Rf.0.25 same solvent system).

After electrolysis was complete (19 hours) the reaction mixture was concentrated to half its volume, made alkaline and left overnight. The following morning it was extracted with ether and the ether extract worked up as usual. The oily residue (20 g) was dissolved in petroleum ether:methylene chloride::1:1 and chromatographed on a silicic acid (200 g) column made in petroleum ether, and eluted continuously with 2% methanol in methylene chloride. The first fraction (400 ml) was discarded as it contained less polar impurities. An oily product was then eluted which was collected separately and shown to be impure dihydroxy coprostane (150 mg). The next fraction (600 ml) gave an oily residue which was

shown to be a single spot on TLC (Rf.0.64; chloroform:acetone: methanol::9:3:1). The next fraction (150 ml) gave impure DHC (3.0 g) from which pure DHC (1.0 g) was obtained on rechromatography.

The oily residue from both the chromatographic procedures - 9.5 g were crystallized from acetone/water to give white needles (8.4 g).

m.p. = 109-111° Reported 108-110°

$[\alpha]_D^{26}$ = +41.3 (c,0.626;d,0.13) +45

IR (ν) = Fig. 15.

$C_{27}H_{48}O_2$ requires C=80.2, H=11.88; found C=80.14, H=11.89

5β-cholestan-3-one-12α-ol

5β-cholestan-3α,12α-diol (6.07 g) was refluxed with Aluminum-isopropoxide (9.0 g) in dry benzene (200 ml) and dry acetone (175 ml) for eighteen hours. The reaction mixture was acidified with 3N-HCl and the acidic phase extracted twice with benzene. The benzene extract was washed until neutral with water, dried over anhydrous sodium sulphate and evaporated to dryness in vacuum. Chromatography on neutral alumina (3% activated; 300 g) was performed by dissolving the residue in benzene:petroleum ether (1:1) and passing the solution through a column prepared with the same mixture of solvents. The column was then eluted with benzene, and 5% ethyl acetate in benzene. Elution with ethyl acetate (7.5%), (400 ml) gave an oily residue (150 mg)

presumably 5β-cholestan-3,12-dione (Rf.0.78, benzene:ethyl acetate:acetone::10:5:3) which could not be crystallized. Elution with 10%, 15% and 20% ethyl acetate in benzene (200 ml) eluted the pure 3-oxo compound (Rf.0.63 in the same solvent system as above, 3.85 g). Further elution with 20% ethyl acetate in benzene (200 ml) gave impure 3-oxo compound contaminated with compound '0' (Rf.0.46). Further elution with 30% ethyl acetate in benzene gave pure compound '0' which was crystallized from hot petroleum ether. Finally 100% ethyl acetate gave no residue on evaporation and 5% acetone in ethyl acetate (200 ml) and 15% acetone in ethyl acetate (400 ml) gave the starting material (1.9 g). These dihydroxy coprostane fractions were pooled and the residue on crystallization from acetone/water afforded 1.7 g of pure starting material (m.p. 108-111°C with slight softening at 105°C).

5β-cholestan-3-one-12α-ol (3.85 g) was crystallized from petroleum ether in white flakes (3.0 g), while at room temperature with slow crystallization thick needles were deposited. Themother liquor was worked up, recrystallized and a fairly pure second batch of crystals (0.5 g) was obtained.

m.p. = 126-127° Reported 122°

$[\alpha]_D^{26}$ = +42.2 (c,0.474;d,0.1) +51

I R (ν) = Fig. 16.

$C_{27}H_{46}O_2$ requires C=80.5, H=11.5; found C=79.96, H=11.53

Cholest-4-en-3-one-12∝-ol

A solution of 5β-cholestan-3-one-12∝-ol (2.0 g) and selenium dioxide (1.6 g) in ethanol (400 ml) was kept at 60°C and the reaction was monitored on fluorescent TLC plates every twenty-four hours (solvent system benzene:ethyl acetate:: 1:1). For the first three days SeO_2 (0.5 g) was added and the reaction was slow. On the fifth day another 0.5 g SeO_2 was added and the reaction was stopped after 148 hours, when 50% conversion to a U.V. absorbing product (Rf.0.48, solvent system same as above)less mobile than the starting material (Rf.0.58) was observed on TLC.

The reaction mixture was filtered to remove precipitated black selenium, evaporated and the residue dissolved in ether. The ether solution was washed with IN-HCl, dried over anhydrous sodium sulphate and evaporated. The residue (2.0 g) was dissolved in benzene and chromatographed on deactivated (5%) neutral alumina (150 g). The column was eluted with increasing amounts of ethyl acetate in benzene so that 5% (150 ml) and 10% (130 ml) removed all traces of less polar impurities (total 175 mg). The last 20 ml of 10% and then 15% ethyl acetate eluates afforded fairly pure starting material (1.03 g). Then 20% and 25% ethyl acetate in benzene (150 ml each) gave fairly pure cholest-4-en-3-one-12∝-ol (0.69 g). In spite of rechromatography, charcoal treatment, the material could not be completely freed from traces of colloidal selenium. Finally on crystallization

from acetone/water cholest-4-en-3-one-12α-ol (0.5 g) was obtained. Identity was established by comparison with the known cholest-4-en-3-one-12α-ol obtained from an independent route by mixed melting point and co-chromatography.

m.p. = 150-152.5° Reported 142-143°

λ max = 242 mμ

$(\alpha)_D^{27}$ = +96.38 (c,0.332;d,0.16) +86

IR (ν) = Fig. 17.

$C_{27}H_{44}O_2$ requires C=81.00, H=11.0; found C=81.22; H=11.03

Further elution of the column with 25% ethyl acetate afforded 50 mg of cholest-1,4-diene-3-one-12α-ol, which was recrystallized from acetone/water.

m.p. = 196-197.5°

$(\alpha)_D^{25.5}$ = +40

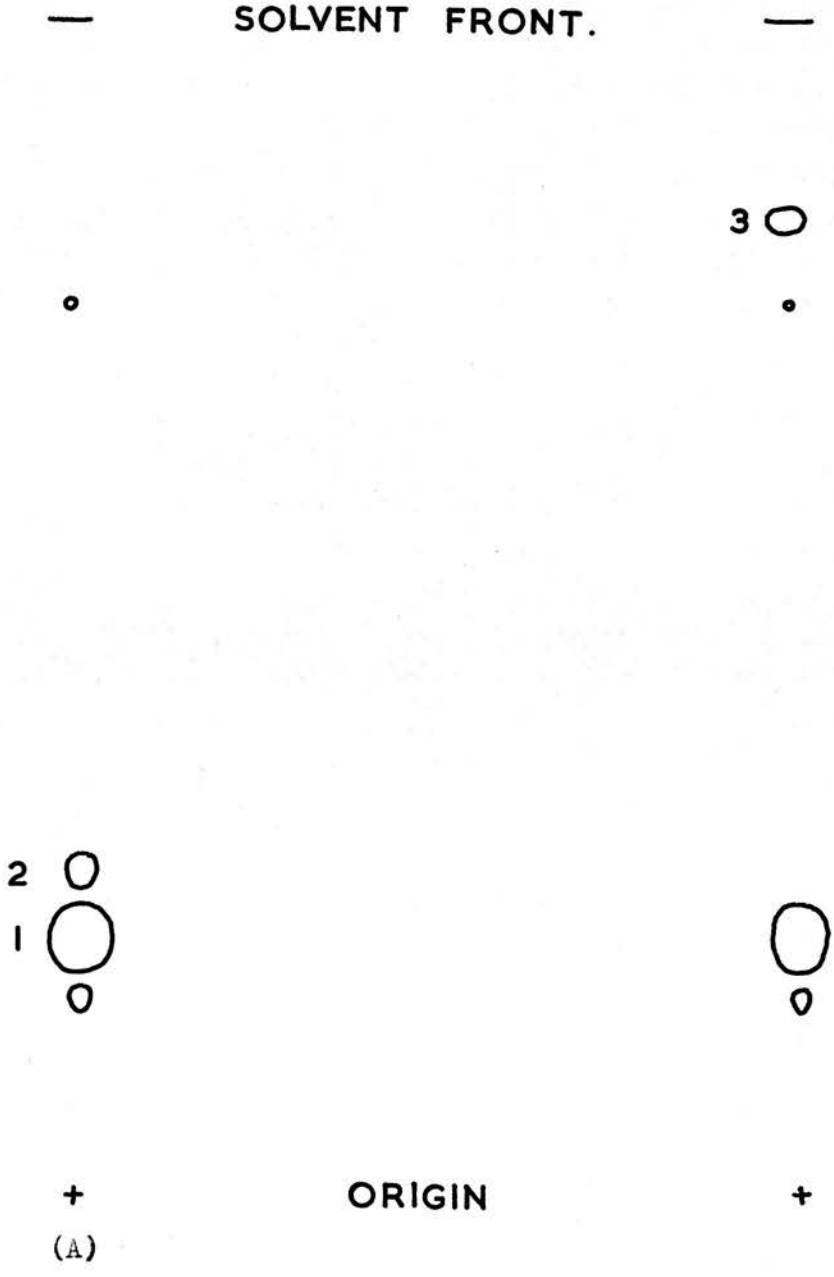
λ max = 246 mμ; Figure 18a.

IR (ν) = Fig. 18.

$C_{27}H_{42}O_2$ requires C=81.4, H=10.55; found C=81.21, H=10.67

Cholest-5-en-3β,12α-diol (12α-hydroxycholesterol)

Enol-acetylation of cholest-4-en-3-one-12α-ol (0.5 g) was accomplished by dissolving it in benzene (40 ml) containing p-toluene sulphonic acid (70 mg) and isopropenyl acetate (2 ml), and slowly distilling about 30 ml of the solvent in three and a half hours. At the end of the first and second hour 1 ml of isopropenyl acetate was added. The solution was evaporated to dryness and the residue dissolved in ether containing a few drops of pyridine. The ether extract was



The reaction mixture after reduction with LiAlH .

(A) after refluxing with methanolic hydrochloric acid.

1= Cholest-5-en-3 ,12 -diol.
2= Cholest-4-en-3 ,12 -diol, and
Cholest-4-en-3 ,12 -diol.
3= Cholest-3,5-diene-12 -diol.

washed with 5% sodium carbonate and then with water. The ethereal residue was dissolved in redistilled ethanol (100 ml) and after cooling to 5°C it was added to a solution of sodium borohydride (1 g) in 25 ml 70% ethanol and the mixture stored overnight at 5°C. The solution was then heated to boiling, 20 ml 5% aqueous sodium hydroxide was added, refluxed for half an hour and then most of the ethanol evaporated in vacuo. There was no hydrolytic product on such treatment. After acidification and extraction with ether, the dry residue was dissolved in ethanol (35 ml) containing 3 drops of conc. hydrochloric acid and refluxed for one hour to dehydrate any 4-ene-3-ols formed to cholest-3,5-diene-12α-ol. Under such a mild treatment 12α-hydroxy or 5-ene-3-ol groupings were unaffected.

The ethanol/solution was concentrated to about 1/4th of its volume. Excess ether was added and the ether layer washed with sodium bicarbonate solution and worked up. The dry residue was reduced with $LiAlH_4$ (0.4 g) in THF to remove the acetoxy group formed at C_{12} and the reduction mixture then worked up. The residue was dissolved in benzene and chromatographed on deactivated (5% water) neutral alumina (60 g) and eluted with increasing amounts of ethyl acetate in benzene. Benzene - 2% ethyl acetate fractions gave the dehydration product i.e. cholest-3,5-diene-12α-ol (Rf.0.71 in benzene:ethyl acetate:: 1:1) while 20% and 25% ethyl acetate in benzene furnished cholest-5-en-3β,12α-diol (Rf.0.38). Crystallization from

benzene afforded pure 12 -hydroxycholesterol in white needles.

m.p. = 152-153.5° Reported 149-150°

$[\alpha]_D^{26}$ = -28 (c,0.55;d,0.08) -27

IR (ν) = Fig. 19.

$C_{27}H_{46}O_2$ requires C=80.5, H=11.4; found C=80.71, H=11.48.

Cholest-3,5-diene-12α-ol was crystallized from chloroform-methanol in white needles.

m.p. = 119-121°

$[\alpha]_D^{26}$ = -84.9

IR (ν) = Fig. 20.

λ_{max} = 229, 235, 244. Figure 20a.

$C_{27}H_{44}O$ requires C=84.34, H=11.45; found C=84.71, H=11.69.

Cholest-4-en-3-one-12α-ol dinitrophenyl hydrazone

5β-cholestan-3-one-12α-ol (0.5 g) was dissolved in glacial acetic acid (6 ml) and bromine solution (0.65 ml) prepared by diluting 0.2 ml bromine with 2 ml acetic acid, was added dropwise at room temperature. The bromine colour was immediately discharged and after standing for half an hour, acetic acid was removed in vacuo, and the residue redissolved in acetic acid (20 ml), 2,4-dinitrophenyl hydrazine (300 mg) added and the mixture warmed under nitrogen to dissolve the hydrazine. After a few minutes heating the hydrazone started to crystallize out and heating was interrupted after an additional three minutes. Cooling the solution overnight under nitrogen deposited a coloured hydrazone. Excess chloroform was added to dissolve all the hydrazone, and the

chloroform solution was washed with water, concentrated and crystallized after adding ethanol. Filtration and washing with ethanol afforded pure dinitrophenyl hydrazone (430 mg Rf.0.82 in benzene:ethyl acetate::1:1).

m.p. = 240-241.5° Reported 237-239°

$CHCl_3$

λ_{max} = 394 mμ

IR (ν) = Fig. 21.

$C_{33}H_{48}N_4O_5$ requires N=9.6; found N=9.02%

Cholest-4-en-3-one-12α-ol

A chloroform (30 ml) solution of the hydrazone (420 mg) was mixed with pyruvic acid (13 g) and 2.5 ml, 5.5 N-hydrogen bromide in glacial acid. Pyruvic acid formed a separate upper layer and therefore the mixture was shaken occasionally while it was warmed between 50-60°C for five hours. It was then diluted with more chloroform and pyruvic acid or its hydrazone removed by washing the solution with aqueous sodium carbonate. The coloured residue from chloroform was chromatographed on deactivated (5%) neutral alumina (50 g) as before. Elution with 15% ethyl acetate afforded cholest-4-en-3-one-12α-ol (100 mg). A slight yellow colour of the residue was decolorised by treatment with activated charcoal in ether. Crystallization of the ethereal residue from methanol-water furnished pure cholest-4-en-3-one-12α-ol (80 mg).

m.p. = 151-153° Reported 142-143°

$C_{27}H_{44}O_2$ requires C=81.00, H=11.0; found C=81.32, H=11.1

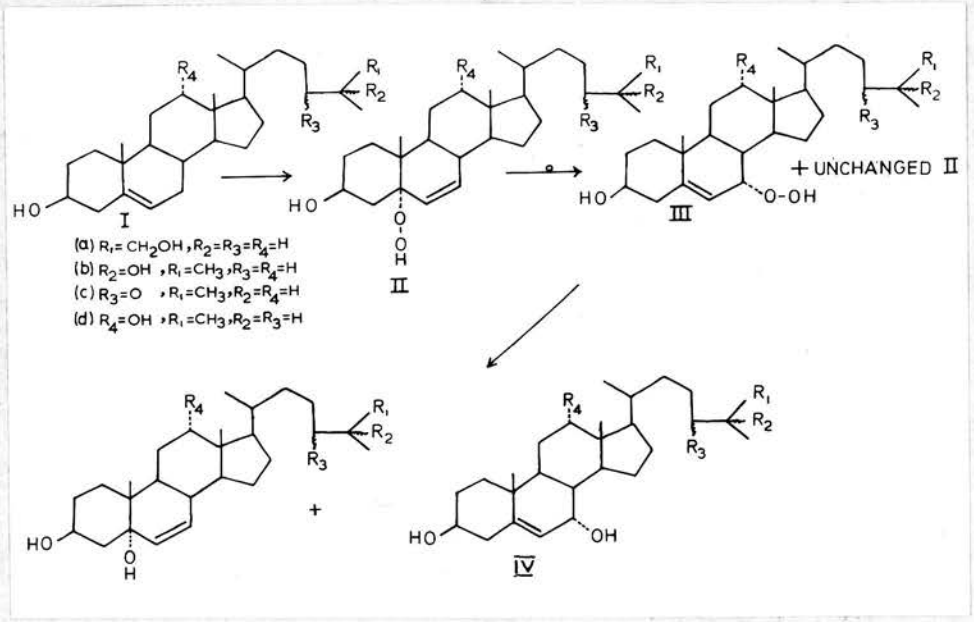

HO
I
(a) $R_1 = CH_2OH$, $R_2 = R_3 = R_4 = H$
(b) $R_2 = OH$, $R_1 = CH_3$, $R_3 = R_4 = H$
(c) $R_3 = O$, $R_1 = CH_3$, $R_2 = R_4 = H$
(d) $R_4 = OH$, $R_1 = CH_3$, $R_2 = R_3 = H$
HO
O
OH
II
HO
O-OH
III
+ UNCHANGED II
HO
O
H
+
HO
OH
IV

Figure. 18.

TRIOLS

Cholest-5-en-3β,7α,26-triol

PREPARATION

(i) In chloroform.

A solution of cholest-5-en-3β,26-diol (250 mg, for preparation refer section I) in chloroform (50 ml; dry and alcohol free) was photo-oxygenated with haematoporphyrin methyl ester (10 mg) for ten hours.

The reaction was followed on thin layer chromatoplates (TLC) every hour. Two spots of the sample mixture were put on the fluorescent TLC plates, well separated from one another, and run in a solvent system (benzene:ethyl acetate:acetone:: 10:5:5). After drying off the solvent the plate was viewed against a U.V. lamp, but no dark spot was located. Half of the plate was sprayed with 10% KI in 20% aqueous ethanol acidified with acetic acid, and the other half was sprayed with phosphotungstic acid (PTA). Hydroperoxides give a yellow spot (Rf.0.5) of liberated iodine with KI-solution after heating the plate at 120°. The total number of products i.e. starting material, desired 7α-hydroperoxide and decomposition products of hydroperoxide such as hydroxy and ketonic compounds were seen on the PTA-sprayed side. This reagent gave a characteristic pink colour with hydroperoxides, flesh colour darkening to chocolate brown with cholest-5-en-3β,26-diol (Rf.0.7) and a blue colour with cholest-5-en-3β,7α,26-triol, cholest-6-en-3β,5α-26-triol or cholest-5-en-3β,7β,26-triol.

No distinction between the latter two compounds could be made, because both had the same mobility and colour response with PTA on TLC.

The photosensitizer was removed by treatment with charcoal and filtration. The clear filtrate was taken to dryness under reduced pressure at 40°C, and reduced with $LiAlH_4$ in tetrahydrofuran (THF). Excess hydride was decomposed with water and the product extracted with ether several times after the addition of a saturated solution of potassium sodium tartrate (Rochelle's Salt) (10 ml). Thin layer chromatography showed incomplete reduction, hence the product was reduced again. The second reduction was almost complete and there were three main spots, the least mobile (Rf.0.3) being the 7α-isomeric triol, and the second more mobile blue spot was deduced to be cholest-6-en-3β,5α-26-triol (Rf.0.4), the third being the starting material (Rf.0.7).

CHROMATOGRAPHY

The desired 7α-isomeric triol was separated on preparative TLC-plates. The plates were run thrice for forty-five minutes in benzene:ethyl acetate (1:2) and for each subsequent run the plates were air-dried. In this way the two main reduction products were separated sufficiently apart (Rfs.0.37 and 0.54). Both sides of each plate were sprayed with PTA and the areas corresponding to the cholest-5-en-3β,7α,26-triol was scraped off and extracted from the silica gel by means of methanol. The solution was

evaporated using/the water pump and the residue on crystallization from methanol and water afforded an amorphous powder (30 mg).

Physical characteristics

m.p.	= 218°	Reported 168-172	
$[\alpha]_D^{26}$	= -77.3	$[\alpha]_D^{24}$	= -79

IR (ν) = Fig. 22.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=77.31, H=11.03.

(ii) In pyridine solution

Cholest-5-en-3β,26-diol (1.0 g) was dissolved in dry pyridine (100 ml) containing haematoporphyrin (14 mg; and after sixteen hours an additional 6 mg) and the solution poured into a photo-oxygenation tube. Oxygen was bubbled in a steady stream for twenty-four hours under illumination. The reaction was monitored on fluorescent TLC-plates as described earlier. After nineteen hours no substantial decrease in the amount of starting material was observed. At the end of twenty-four hours there were four spots on the plate, one having the same mobility as cholest-5-en-3β,26-diol, the second and major spot being cholest-6-en-3β,26-diol-5α-hydroperoxide (yellow spot with KI-solution) followed by two less mobile spots (blue with PTA) in the region of cholest-5-en-3β,7α,26-triol and cholest-6-en-3β,5α,26-triol. The photo-oxygenation mixture was then treated with charcoal to remove the catalyst, and the pale yellow solution was concentrated in a rotary evaporator at 45°C. Traces of

pyridine were removed by treatment with chloroform. The dry powder thus obtained was suspended in 100 ml chloroform (acid, alcohol free) and left at room temperature for thirty hours with occasional shakings, filtered, and the filter paper washed with hot chloroform. The filtrate was then evaporated on a water pump at 40°C and the residue dried under high vacuum for several hours. A TLC-plate at this stage showed no further decomposition products. Reduction of the isomerised product with $LiAlH_4$ was performed in THF. The $LiAlH_4$ (0.5 g) was suspended in 20 ml freshly distilled THF and a solution of isomerised product in the same solvent (5 ml) was dropped in carefully. After standing for one hour at room temperature the mixture was refluxed for half an hour, cooled and excess of $LiAlH_4$ decomposed by careful addition of water. A saturated solution of potassium sodium tartrate was added and the reduction products extracted several times with ether and worked up as usual. The residue, when run on TLC-plates still showed substantial amounts of unreduced hydroperoxide and therefore the product was reduced again. This time the reduction product showed only a trace of a hydroperoxide spot on TLC. The reduction products were comparable to those obtained with direct photo-oxygenation in chloroform.

Chromatography of the product on neutral alumina (with or without water) did not separate cholest-6-en-3β,5α,26-triol from cholest-5-en-3β,7α,26-triol. All the fractions

containing the cholest-5-en-3β,7α,26-triol were contaminated with cholest-6-en-3β,5α,26-triol. Those fractions which had a little impurity of cholest-6-en-3β,5α,26-triol were pooled and the residue crystallized several times from ethyl acetate, but the crystals were mixed with the more mobile 5α-triol. Cholest-5-en-3β,7α,26-triol was then purified in the following manner.

The mixture (0.5 g) as white amorphous powder was transferred to a boiling tube provided with a drawn out long neck. Acetone in petroleum ether (50:50) (10 ml) was poured in, the glass tube was then sealed up after adding a few glass beads, and subjected to equilibration between solid and solution phases on an oscillating shaker in a cold room for a fortnight. The more soluble cholest-6-en-3β,5α,26-triol went into solution and the less soluble cholest-5-en-3β,7α, 26-triol initially dissolved was precipitated. The tube was opened, centrifuged, and the clear supernatant containing mainly the 5α-triol was pipetted off. Five to six such treatments gave pure cholest-5-en-3β,7α,26-triol (240 mg).

The amorphous powder was then crystallized from hot ethyl acetate as white semicrystalline needles. A mixed melting point with the previous sample did not depress the melting point and confirmed the identity of the two products.

m.p. = 218°

SEPARATION OF CHOLEST-5-EN-3β,7α,26-TRIOL AND CHOLEST-6-EN-3β,5α,26-TRIOL AFTER ACETYLATION

A mixture of the isomeric triols (300 mg) was acetylated in pyridine (5 ml) with acetic anhydride (0.5 ml) at room temperature for four days and worked up as usual. The ethereal residue (320 mg) of triacetates showed a single spot on TLC plates in two different solvent systems (Rf.0.7 in benzene:ethyl acetate::1:1, and 0.42 in benzene:petroleum ether:ether::1:1:1 or 0.3 in 2:2:1) and was chromatographed on alumina (Spence '0' type; 15 g). The triacetate mixture was dissolved in petroleum ether and passed through the column, which was eluted with increasing amounts of benzene in petroleum ether i.e. 10, 20, 30, 40, 50, 75 per cent benzene, followed by pure benzene and finally with 2% ethyl acetate in benzene. All these fractions (25 ml) left no residue on evaporation. The ethyl acetate in benzene (5%) fraction gave 100 mg of residue which on hydrolysis with 5% NaOH in aqueous methanol overnight at room temperature gave pure cholest-5-en-3β,7α,26-triol. Further elution with 7.5% and 10% ethyl acetate in benzene gave 85 and 30 mg of residue respectively, which on hydrolysis gave a mixture of the two isomeric triols. Further elution resulted in hydrolysis of the product on the column, therefore the column was washed with methanol and the total isomeric mixture was reacetylated and rechromatographed on neutral alumina (10 g) and eluted as above. The ethyl acetate (5%) fraction on

hydrolysis as above gave pure cholest-5-en-3β,7α,26-triol (Rf.0.26 in benzene:ethyl acetate:acetone::10:5:5). The next 10% fraction on hydrolysis gave a mixture and finally the 20% ethyl acetate in benzene fraction on hydrolysis gave pure cholest-6-en-3β,5α,26-triol (Rf.0.32). The product was crystallized from methanol-water as white needles. Very little hydrolysis was encountered on neutral alumina.

The identity of cholest-5-en-3β,7α,26-triol was established by mixed melting point and co-chromatography.
Melting point and mixed melting point = 218° - no depression.

When a solution of cholest-6-en-3β,5α,26-triol (20 mg) in chloroform (3 ml) was treated with MnO_2 (0.2 g) for eight hours with occasional shaking no ultra-violet absorbing material was detected using fluorescent TLC-plates. Under the same condition cholest-5-en-3β,7β-diol was completely converted to the more mobile ultra-violet absorbing material i.e. cholest-5-en-3β-ol-7-one.

Physical characteristic of cholest-6-en-3β,5α,26-triol:-

m.p. = 205-207.5°

$(\alpha)_D^{26}$ = -10

IR (ν) = Fig.23.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=77.16; H=11.2

Cholest-5-en-3β,7α,24ξ -triol

Cholest-5-en-3β-ol-24-one.

Cholest-5-en-3β-ol-24-one-3β-acetate (1.0 g m.p. 130-132° - Organon) was hydrolysed with K_2CO_3 (0.7 g) in 10% aqueous methanol at room temperature for twelve hours. The mixture was warmed for one hour and the free sterol precipitated with water and left for crystallization in cold. Filtration and crystallization from benzene:methanol afforded pure cholest-5-en-3β-ol-24-one (0.9 g; m.p. 137-139°; Rf.0.58 in benzene:ethyl acetate:acetone::10:5:3).

Cholest-5-en-3β-ol-24-one (0.9 g) was dissolved in dry pyridine (100 ml) and photo-oxygenated with haematoporphyrin (14 mg) for twenty-four hours as before. Most of the starting material was converted to another product, presumably cholest-6-en-3β-ol-5α-hydroperoxy-24-one (yellow spot with KI solution; Rf.0.45). The photo-oxygenation mixture was worked up as before. Isomerization was achieved by leaving at room temperature for twenty-four hours in chloroform (150 ml) and the product reduced twice with $LiAlH_4$ (0.9 g) in THF, because the first reduction was incomplete. There were two main spots of the reduction product, the least mobile being cholest-5-en-3β,7α,24ξ -triol (Rf.0.2) and the more mobile (Rf.0.3) presumably cholest-6-en-3β,5α,24ξ -triol, in almost equal proportions. The reduction mixture was worked up as before.

CHROMATOGRAPHY

The residue from the ether extract was chromatographed on deactivated neutral alumina (4% water; 50 g). A suspension of the residue in ether (4 ml) was passed through the column prepared in petroleum ether. The flask was washed thrice with 2 ml ether each time and the washings were transferred on to the column, which was then washed with 100 ml petroleum ether. Acetone in petroleum ether 5% and 10% removed the more mobile impurities and the 15% acetone fraction on evaporation afforded cholest-5-en-3β,24ξ-diol (50 mg, m.p. 172°). Acetone in petroleum ether fractions (20 and 25%) removed other unidentified oily products, which were more mobile than cholest-6-en-3β,5α,24ξ-triol. The latter was expected to have a similar mobility to cholest-5-en-3β,7β,24ξ-triol by analogy with cholest-6-en-3β,5α-diol and cholest-5-en-3β,7β-diol. The 30% acetone in petroleum ether fraction afforded cholest-6-en-3β,5α,24ξ-triol (187 mg). The 35% acetone in petroleum ether fraction gave slightly impure cholest-5-en-3β,7α,24ξ-triol (250 mg), and the 40% and 50% acetone in petroleum ether fractions afforded pure cholest-5-en-3β,7α,24ξ-triol (210 mg).

The 35% acetone in petroleum ether fraction was rechromatographed on deactivated neutral alumina (25 g) as above. The 30% acetone in petroleum ether fraction gave cholest-6-en-3β,5α,24ξ-triol (50 mg) followed by a mixture of 7α- and 5α-epimeric hydroxy-triols (100 mg) in the 35% acetone

in petroleum ether fraction. In the 40%, 50% and 60% acetone in petroleum ether pure cholest-5-en-3β,7α,24ξ-triol (140 mg) was obtained.

Total yield of cholest-5-en-3β,7α,24ξ-triol = 350 mg.

" " " cholest-6-en-3β,5α,24ξ-triol = 237 mg.

Mixture of cholest-5-en-3β,7α,24ξ-triol and cholest-6-en-3β,5α,24ξ-triol = 100 mg.

PHYSICAL CHARACTERISTICS

(a) Cholest-5-en-3β,7α,24ξ-triol.

m.p. = 204° (previous softening at 172°)

$(\alpha)_D^{26} = -76.8$

IR (ν) = Fig. 24.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=77.43, H=10.95.

Cholest-5-en-3β,7α,25-triol

Preparation

Cholest-5-en-3β,25-diol (1.0 g) was dissolved in anhydrous pyridine (100 ml) containing 14 mg haematoporphyrin and 6 mg more added after nine hours. The mixture was photo-oxygenated for nineteen hours. Analysis by TLC showed one main spot corresponding to cholest-6-en-3β,25-diol-5α-hydroperoxide (Rf.0.34 in solvent system benzene:ethyl acetate:acetone:: 10;5;5) together with starting material (Rf.0.5) and traces of cholest-5-en-3β,7α,25-triol and cholest-6-en-3β,5α,25-triol (Rfs.0.17 and 0.24 respectively).

The reaction mixture was taken to dryness as before and the residue suspended in dry chloroform (150 ml) and kept for two days at room temperature for isomerization of the 5α-hydroperoxide to the 7α-hydroperoxide. The mixture was then filtered and evaporated as before. The residue after drying overnight under high vacuum was reduced with $LiAlH_4$ (0.8 g) as usual, but increasing the reflux time for one hour. Again TLC showed incomplete reduction of the hydroperoxide and hence the reduction was repeated with a fresh batch of $LiAlH_4$. On this occasion there was no trace of a spot on TLC corresponding to the hydroperoxide. There were again two main spots of the reduction product corresponding to cholest-5-en-3β,7α,25-triol and cholest-6-en-3β,5α,25-triol in almost equal proportion. Acetone (3 ml) was added to the semicrystalline residue (0.98 g) and left for twelve hours at $0^{o}C$. The residue was

then transferred to a boiling tube provided with a narrow glass tube. Acetone (8 ml) and glass beads were added and after sealing, the glass tube was left on a slow shaker for a fortnight in a cold room to equilibrate between the solid and solution phases. The sealed tube was then carefully cut open, centrifuged, and the acetone solution sucked off by means of a Pasteur pipette. This acetone solution showed by TLC, mainly the 5α-isomeric triol and the starting material i.e. cholest-5-en-3β,25-diol. The 60% acetone in petroleum ether solution was used for solid-solution equilibration purpose, and the process repeated several times. The amorphous white solid was still impure. This method of purification was very slow and therefore further purification was achieved through acetylation and chromatography of the acetylated product.

CHROMATOGRAPHY

(a) The acetone solution was evaporated and the residue (200 mg) was acetylated in pyridine and acetic anhydride at room temperature for twelve hours. The reaction mixture was worked up as usual and the acetylated product chromatographed on neutral alumina (15 g). The chromatographic column was prepared in petroleum ether and the mixture dissolved in 40% benzene in petroleum ether was adsorbed on the column which was then eluted with increasing amounts of benzene in petroleum ether. Ethyl acetate in benzene 4% gave cholest-5-en-3β,25-diacetate (8 mg) which on hydrolysis

gave cholest-5-en-3β,25-diol. Ethyl acetate in benzene (8%) fraction after hydrolysis gave material with the same mobility as cholest-5-en-3β,7α,25-triol (3 mg). The 12% ethyl acetate in benzene fraction after hydrolysis gave traces of both 7α-and 5α-isomeric triols. The 20% ethyl acetate in benzene fraction after hydrolysis gave cholest-5-en-3β,25-diol (28 mg). The 30% ethyl acetate in benzene fraction gave mixtures of two isomeric triols after hydrolysis (70 mg) while the 40% ethyl acetate in benzene fraction gave impure cholest-6-en-3β,5α,25-triol (8 mg). Pure ethyl acetate gave cholest-6-en-3β,5α,25-triol (85 mg).

Physical characteristics of cholest-6-en-3β,5α-25-triol.

m.p. = 232° (softening at 172; shrinking and recrystallization at 192).

$[\alpha]_D^{26}$ = -18

IR ( ) = Fig. 25.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C= 69.88 H= 10.87.

(b) The amorphous white powder was acetylated and the acetylated product (780 mg) was chromatographed on neutral alumina (50 g) as described above. The 4% ethyl acetate in benzene fraction gave impure cholest-5-en-3β,7α,25-triol (210 mg) which was purified on preparative TLC and cholest-5-en-3β,7α,25-triol was obtained. The ethyl acetate in benzene fraction (8%) (140 mg) gave relatively pure cholest-5-en-3β,7α,25-triol after hydrolysis from which a pure product was obtained on crystallization from methanol-water.

The 15% ethyl acetate in benzene fraction gave a residue (110 mg) from which cholest-6-en-3β,5α,25-triol was obtained on crystallization from ether and ethanol-water. The 20% ethyl acetate in benzene fraction (180 mg) gave a mixture of both isomeric triols from which the desired cholest-5-en-3β,7α,25-triol was obtained by preparative TLC-plates. The 25% ethyl acetate in benzene fraction yielded 40 mg pure cholest-5-en-3β,7α,25-triol on crystallization from methanol-water. The 30% and 40% ethyl acetate in benzene fractions gave a residue (25 mg) which was discarded, while the 50% and 60% ethyl acetate in benzene gave pure cholest-6-en-3β, 5α,25-triol after hydrolysis.

The pattern of chromatography is complicated because both tertiary hydroxyl groups at C-5 and C-25 were only partially acetylated. The acetylation was performed at 4°C but the reaction mixture was left for a considerable time.

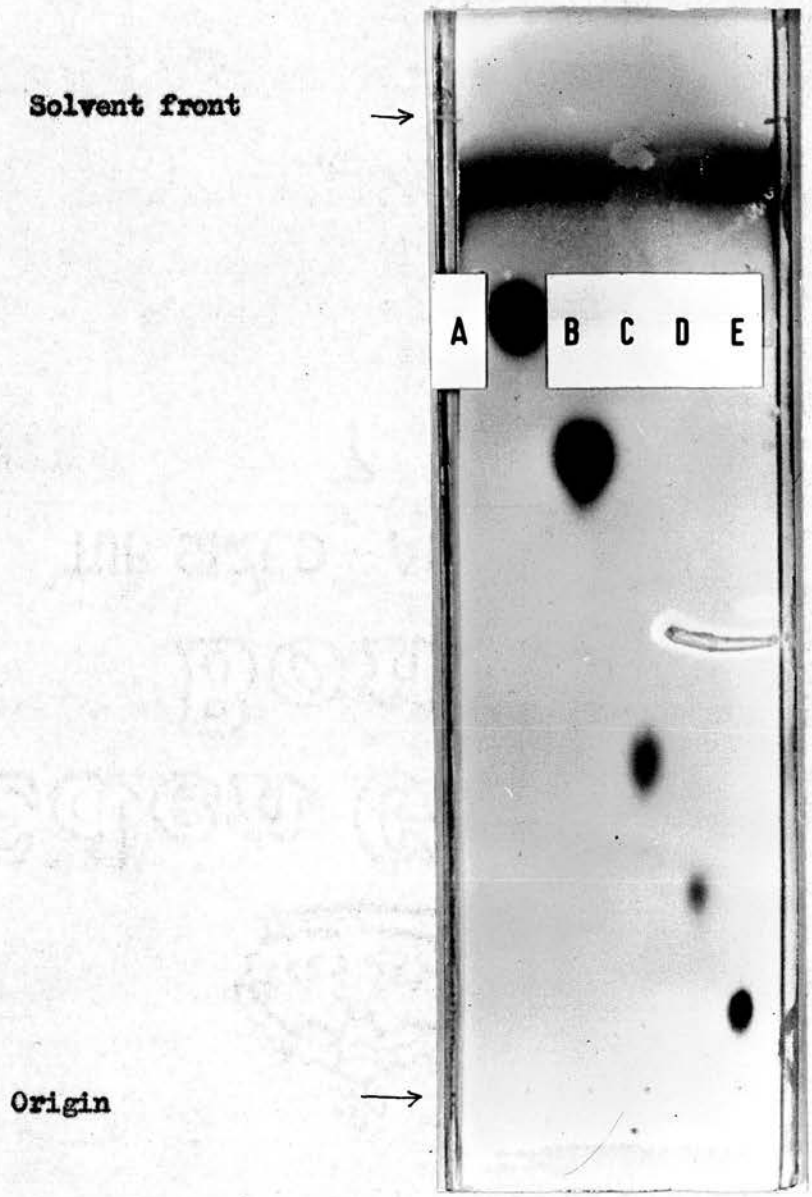
Physical characteristic of cholest-5-en-3β,7α,25-triol.

m.p. = 229° (softening and recrystallization between 215-220°)

$[\alpha]_D^{26}$ = -89.1

IR (ν) = Fig. 26.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=77.97, H=10.81



Thin layer chromatogram showing relative mobilities of the following compounds in benzene : ethyl acetate :: 7 : 13.

(A) Cholest-5-en-3β-ol.
(B) Cholest-5-en-3β,12α-diol.
(C) Cholest-5-en-3β,7α-diol.
(D) Cholest-6-en-3β,5α,12α-triol.
(E) Cholest-5-en-3β,7α,12α-triol.

Cholest-5-en-3β,7α,12α-triol

Cholest-5-en-3β,12α-diol (50 mg) was photo-oxygenated in pyridine (Ca. 10 ml) in the presence of haematoporphyrin (Ca. 2 mg) as usual and the reaction followed on TLC. After approximately ten hours all of the starting material (Rf.0.76) was converted to a more polar product (Rf.0.61 in benzene: ethyl acetate::7:13). The reaction mixture was worked up as before and the product isomerized in chloroform for eighteen hours. The chloroform solution was taken to dryness and the residue reduced with $LiAlH_4$ solution in THF (10 ml). The reduction mixture was refluxed for 1.5 hours and as TLC showed incomplete reduction hence more $LiAlH_4$ was added and the mixture kept at reflux temperature for another 0.5 hour. Again TLC revealed incomplete reduction. The reaction mixture was then worked up as before and the ethereal residue, cholest-5-en-3β,7α,12α-triol and cholest-6-en-3β,5α,12α-triol were separated on preparative TLC plates using benzene:ethyl acetate:acetone::10:5:5. Crystallization from methanol-water afforded pure cholest-5-en-3β,7α,12α-triol and pure cholest-6-en-3β,5α,12α-triol.

Cholest-5-en-3β,7α,12α-triol. m.p. 193°

Cholest-6-en-3β,5α,12α-triol. m.p. 242° (decomp.)

No other characteristics could be determined due to paucity of the material.

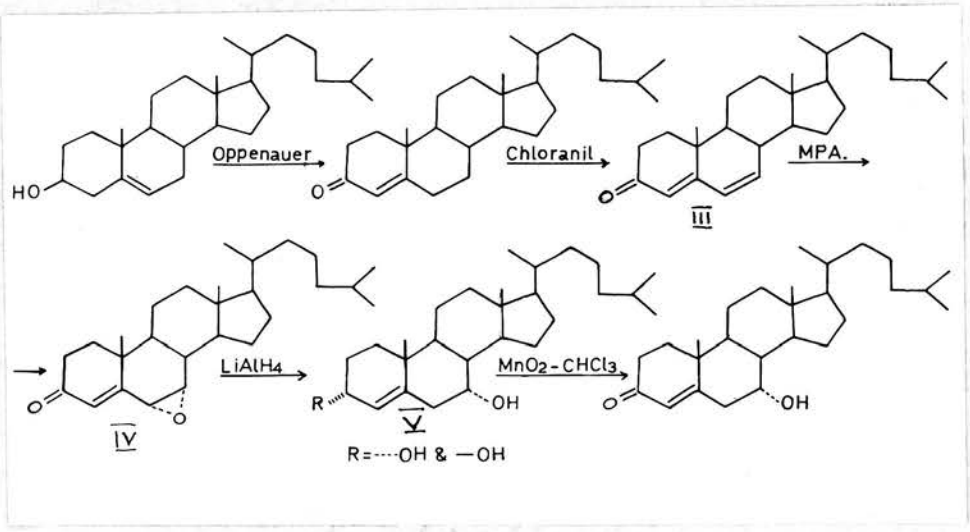

HO
Oppenauer
O
Chloranil
O
III
MPA.
LiAlH4
O
O
IV
R
OH
V
R=----OH & —OH
MnO2 - CHCl3
O
OH

Figure. 19.

Cholest-4,6-diene-3-one

Cholest-4-en-3-one (11 g) and chloranil (40 g) were refluxed in t-butyl alcohol (350 ml) under nitrogen for three hours. After cooling the reaction mixture, the unreacted chloranil was filtered off and washed with ether. The filtrate and ether washings were combined and evaporated, the residue was dissolved in ether, washed with water, 5% sodium hydroxide and finally washed neutral with water. The other solution was dried over anhydrous sodium sulphate and evaporated under vacuum giving 9.0 g of a yellow product which was then chromatographed on silicic acid.

The residue was dissolved in petroleum ether and passed through the column (125 g silicic acid) washing with an excess petroleum ether until a yellow band moved down the column and separated clearly. Ether in petroleum ether (5% - 250 ml) eluted the residual yellow reagent. Ether in petroleum ether (8%and 10%) gave pure cholest-4,6-diene-3-one (8.0 g). A hot solution of methanol was seeded and the flask wrapped in a cloth for slow cooling to room temperature, when most of the product cryatallized. Further crystallization was accomplished in the cold. Filtration afforded 7.4 g flat needles.

m.p. = 80-81° Reported 81.5°

$(\alpha)_D^{27}$ = +35 +36

λ_{max} = 282 mμ

$C_{27}H_{42}O$ requires C=84.75, H=11.07; found C=84.40, H=11.21

(A) Epoxidation at 5°C in ether

Cholest-4,6-diene-3-one (5.73 g) was dissolved in an ether solution of monoperphthalic acid (36 ml; 1.26N; 121 mg/ml) and the total volume made up to 45 ml. The solution was kept at 5°C and the first sample for TLC was taken out after forty-eight hours when 50% conversion to the less mobile epoxy compound was noted. More monoperphthalic acid (10 ml) was added and the mixture left over for another twelve hours. A conversion of 70-75% was detected by TLC. The reaction mixture was diluted with ether and the suspension washed with 5% sodium carbonate. The alkaline ether solution was washed neutral with water, dried over anhydrous sodium sulphate and evaporated to dryness. The residue (5.0 g) was taken up in 10% benzene in petroleum ether and left for twelve hours at room temperature. A crystalline product separated and washed with 10% benzene in petroleum ether. The filtrate and washings were chromatographed on deactivated (4%) neutral alumina (85 g) with increasing amounts of benzene. Benzene 25% in petroleum ether eluted a less polar substance and 35% benzene in petroleum ether gave a mixture of dienone and less polar product. Benzene 45% in petroleum ether eluted pure starting material and this was followed by fractions which gave impure epoxide (1.8 g). Crystallization from methanol afforded pure needles (1.2 g).

m.p. = 137.5-138.5° Reported 138.5-139°

$(\alpha)_D^{26}$ = -56.6 (c,0.406;d,0.115) -59

IR(ν) = Fig. 27.

λ max = 242 mμ

$C_{27}H_{42}O_2$ requires C=81.4, H=10.5; found C=81.19, H=10.46

(B) <u>At 27° in chloroform-ether</u>

Cholest-4,6-diene-3-one was epoxidized using chloroform (9 ml) and an ether solution of monoperphthalic acid (1.26 N). The reaction mixture was kept at 5°C for five hours and then at 27°C for a further nineteen hours. The reaction product was worked up as before. The residue (5.3 g) was chromatographed on deactivated neutral alumina (3%, 100 g) as above. Benzene 20% and 30% in petroleum ether gave a less polar product. Benzene 40% and 50% furnished impure dienone (1.74 g). Pure benzene and 5% ether in benzene gave almost pure epoxide (1.4 g). Crystallization from methanol afforded needles (1.1 g).

m.p. = 137-138°

λ max = 242 mμ

(C) Dienone (3.3 g) was epoxidized as above in a mixture of chloroform and ether solution of monoperphthalic acid (60% conversion). Unreacted monoperphthalic acid was removed by filtration of the reaction mixture through neutral alumina (50 g). Elution with chloroform (500 ml) gave only 2.2 g of the product which showed more dienone than epoxide. Chromatography on neutral alumina (3%, 50 g) gave 1.1 g starting material and impure epoxide (0.56 g). Crystallization from absolute ethanol gave pure epoxide (0.35 g).

m.p. = 138°

Cholest-4-en-7α-ol-3-one

The foregoing epoxide (400 mg) was reduced with $LiAlH_4$ in THF as usual. The mixture was refluxed for half an hour and after decomposition of excess of hydride with ethyl acetate, a saturated solution of potassium sodium tartrate (Rochelle's salt) was added and the mixture extracted several times with ether. The ether extract was worked up as usual. Chromatography on TLC plates showed two spots corresponding to the cholest-4-en-3β,7α-diol (major spot Rf.0.29; benzene: ethyl acetate:acetone::10:5:3) and cholest-4-en-3α,7α-diol (minor spot Rf.0.22). Infra-red spectrum at this stage showed no carbonyl peak and there were large hydroxyl peaks as expected. Since both 3α- and 3β-allylic hydroxyl groups can be oxidized by means of MnO_2 in chloroform solution to α,β-unsaturated ketone, the mixture of the diols was dissolved in chloroform (40 ml) and manganese dioxide (4 g) prepared according to Mancera (1953) was added. The suspension was kept at room temperature for ten hours and the reaction followed every two hours. After six hours very little starting material was left. The catalyst was filtered off and washed with chloroform to ensure quantitative recovery. The residue from the chloroform solution was dissolved in 5-10 ml of 50% benzene in petroleum ether and the solution chromatographed on deactivated (6%) neutral alumina (15 g) column prepared in petroleum ether. The column was then washed with 50% benzene in petroleum ether followed by 80%

benzene in petroleum ether and then pure benzene. Elution with 5% ether in benzene and 10% ether in benzene gave a residue (300 mg) which was pure on TLC assessment. Crystallization from ether afforded white needles of cholest-4-en-3-one-7α-ol.

m.p. = 183-184° Reported 183.5-184.5°

$(\alpha)_D^{26}$ = +67 +67

λ max = 242 mμ; IR ( ) = Fig. 28.

$C_{27}H_{44}O_2$ requires C=80.95, H=11.1; found C=80.78, H=11.29

15% and then pure ether gave (30 mg) the starting material.

5β-cholestan-3-one-7α-ol

5β-cholestan-3α,7α-diol (DHC) was prepared by electrolytic coupling of chenodeoxycholic acid and isovaleric acid as described by Bergstrom and Krabisch (1957).

5β-cholestane-3α,7α-diol (400 mg) was refluxed with aluminium isopropoxide (800 mg) in dry benzene (15 ml) and acetone (7.5 ml) for three hours. The reaction mixture was acidified with 2N-H_2SO_4 and worked up as usual. Chromatography of the ether residue (390 mg) on deactivated (4%) neutral alumina (25 g) with increasing percentage of ethyl acetate in benzene eluted 5β-cholestan-3-one-7α-ol in 5% and 7.5% ethyl acetate. The residue from these fractions was crystallized from acetone-water (140 mg). Starting material (DHC 130 mg) was recovered from 45% ethyl acetate.

m.p. = 118-120° Reported 121°

$(\alpha)_D^{26}$ = +15.7 +16

IR (ν) = Fig. 29.

$C_{27}H_{46}O_2$ requires C=80.50, H=11.44; found C=80.14, H=11.60

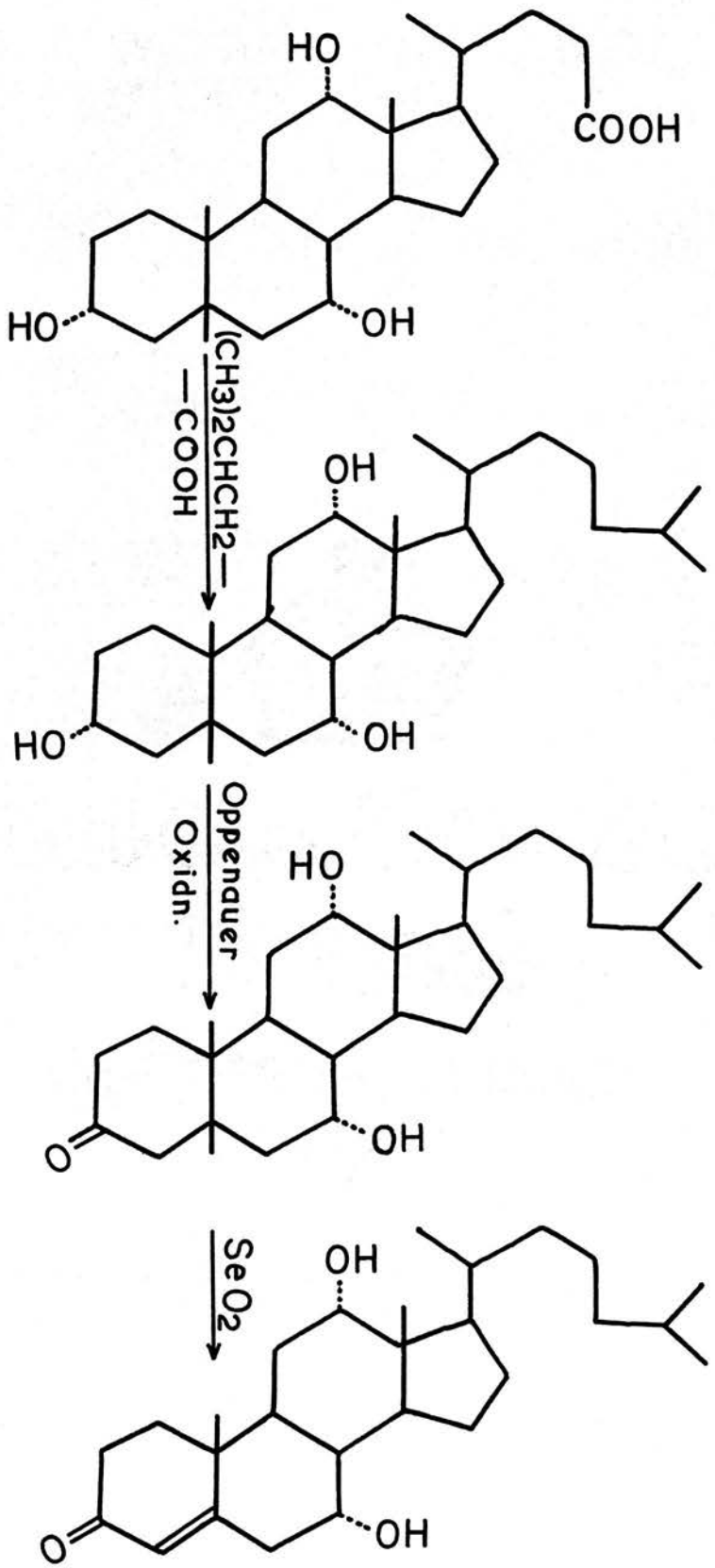

HO
COOH
HO
OH
(CH3)2CHCH2—
—COOH
OH
HO
OH
Oppenauer
Oxidn.
HO
O
OH
SeO2
OH
O
OH

Fig. 20

5β-cholestan-3α,7α,12α-triol: (Trihydroxycoprostane or THC)

THC was prepared by electrolysis for sixteen hours of cholic acid, 15 g (0.05 mole) and isovaleric acid, 67 ml or 61 g (0.6 mole) sodium 2.2 g in methanol 750 ml with 1.25 amp between two platinum electrodes (1" x 2") placed ¼" apart. Thin Layer Chromatography showed only a trace of cholic acid (Rf.0.1, chloroform:acetone:ethanol::54:18:6 drops of acetic acid) and main spot of THC (Rf.0.36).

The mixture was worked up according to Kazuna and Mori (1954). Crystallization of this mixture afforded impure THC (9.1 g) which showed traces of impurities on TLC.

The mother liquor was extracted four times with ether and from the residue (2.7 g) impure THC (0.5 g) was recovered through chromatography on silicic acid.

Crystals (9.1 g) were dissolved in CH_2Cl_2 and the solution chromatographed on a silicic acid column (200 g) prepared in the same solvent and eluted with 3% methanol in CH_2Cl_2. After 700 ml of eluant, each fraction of 100 ml was collected from which fractions III to X gave pure THC on crystallization from acetone-water (5.8 g).

Fraction XI, the mother liquor, and the 0.5 g impure THC were pooled (2 g), dissolved in benzene and chromatographed on alumina (type '0' Spence). The column was eluted with increasing amounts of acetone in benzene. Methanol in acetone (5% and 10%) afforded 0.55 g of pure THC after crystallization from acetone-water.

m.p. = 190–192° Reported 185–186°

$(\alpha)_D^{26}$ = +28.2 (c,0.78;d,0.11) +30.4

IR (ν) = Fig. 30.

$C_{27}H_{48}O_3$ requires C=77.14, H=11.73; found C=77.07, H=12.00

5β-cholestane-3-one-7α,12α-diol

Trihydroxycoprostane (3.3 g) was refluxed with aluminium isopropoxide in dry benzene (200 ml) and acetone (120 ml) for twelve hours. Refluxing was stopped because of vigorous bumping and after cooling the reaction mixture was treated with 3N-H_2SO_4 and the benzene layer separated. The acid layer was extracted twice with benzene and the extracts were combined, washed with 2% Na_2CO_3 solution and then washed neutral with water. The residue was suspended in benzene (50 ml) and poured onto the deactivated (5%) neutral alumina (150 g) column. The container was washed with 5% ethyl acetate in benzene (100 ml) and the extract passed through the column, which was eluted with increasing amounts of ethyl acetate in benzene, collecting 150 ml fractions. Elution with ethyl acetate in benzene (20% and 30%) gave less polar products, (Rf.0.82 and 0.78; chloroform: acetone:methanol::48:12:6) 50% (last 50 ml) and pure ethyl acetate gave the 5β-cholestan-3-one-7α,12α-diol (1.86 g). The column was then eluted with 10%, 20% acetone in ethyl acetate and then pure acetone. Methanol in acetone (20% and 25%) gave pure trihydroxycoprostane - the starting material (0.96 g).

5β-cholestan-3-one-7α,12α-diol (1.86 g) was crystallized from hot ethyl acetate:petroleum ether (1:2, 50 ml) and the pure compound (1.45 g) was obtained. The residue from the mother liquor was recrystallized from ethyl acetate:petroleum ether (1:3, 10 ml) and a second crop of pure crystals obtained.

m.p. = 211-213° Reported 209-210°

$(\alpha)_D^{26}$ = +32 +42.6

IR (ν) = Fig. 31.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=77.92, H=11.14

Cholest-4-en-3-one-7α,12α-diol

(a) A solution of 5β- cholestan-3-one-7α,12α-diol (140 mg) and selenium dioxide (110 mg) in ethanol (35 ml) was left at 60°C for twenty-eight hours. More selenium dioxide (60 mg) was added after twelve hours. At the end of twenty-eight hours, when most of the starting material (Rf.0.55) was converted to the less mobile cholest-4-ene-3-one-7α,12α-diol (Rf.0.39, benzene:ethyl acetate:acetone::10:5:5) the reaction mixture was filtered, evaporated and chromatographed on deactivated (6%) neutral alumina (10 g). The benzene solution was passed through a column which was eluted with 20% ethyl acetate in benzene (50 ml), the last 25 ml giving starting material (26 mg). The first 15 ml of 30% ethyl acetate in benzene fraction (50 ml) further afforded starting material containing traces of cholest-4-en-3-one-7α,12α-diol, and the remaining 35 ml gave rise to cholest-4-en-3-one-7α,12α-diol (74 mg). Crystallization from acetone-water afforded the pure compound (50 mg).

m.p. = 228-229° Reported 228-229°

$[\alpha]_D^{26}$ = +86.2 (c,0.406;d,0.175) +85.0

λ max = 242 mμ

IR ( ) = Fig. 32.

$C_{27}H_{44}O_3$ requires C=77.8, H=10.58; found C=78.36, H=10.64

(b) 5β-cholestan-3-one-7α,12α-diol (300 mg), SeO_2 (270 mg) alcohol (60 ml) were treated as above. More SeO_2 (100 mg) was

added after sixteen and twenty-five hours and the heating was continued for forty-one hours. On TLC there was only a trace of starting material the main product corresponded to cholest-4-en-3-one-7α,12α-diol. The reaction mixture was worked up and chromatographed as above on alumina (25 g). Ethyl acetate 40% in benzene (50 ml) gave 125 mg of cholest-4-en-3-one-7α, 12α-diol containing traces of starting material from which pure cholest-4-en-3-one-7α,12α-diol (77 mg) was obtained after rechromatography. Ethyl acetate 50% in benzene followed by 60% ethyl acetate gave 127 mg of cholest-4-en-3-one-7α,12α-diol.

Total cholest-4-en-3-one-7α,12α-diol (204 mg) was crystallized from acetone-water and pure cholest-4-en-3-one-7α,12α-diol (152 mg) was obtained.

m.p. = 229°

λ max = 242 mμ

IR (ν) = Fig. 32

(c) When 30 mg of coprostane-3-one-7α,12α-diol was refluxed with 30 mg of SeO_2 in 10 ml ethanol, although the reaction was complete in sixteen hours, more of the 1,4-diene-3-one was formed than in the previous two cases, i.e. when the temperature was kept below 65°C.

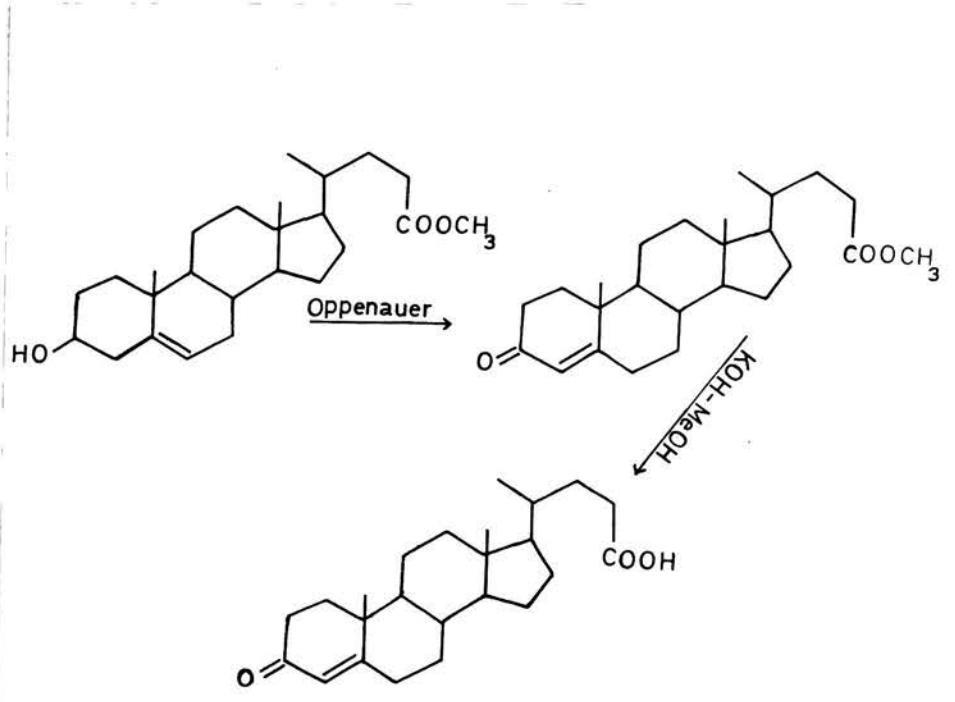


Figure 21.

Methyl 3-keto-chol -4-enate

3β-hydroxy- Δ^5-cholenic acid methyl ester (4.5 g) was refluxed for ten hours with aluminium isopropoxide (6 g) in dry benzene (180 ml) and acetone (100 ml). The reaction mixture was acidified and the benzene layer separated. The acidic phase was extracted twice with benzene. The total benzene extract was washed with water, dried, and evaporated in vacuo. The residue was taken up in petroleum ether and chromatographed on a silicic acid column (50 g) eluting with increasing amounts of benzene. Pure benzene followed by 5% and 10% ether yielded methyl 3-keto-chol -4-enate (3.5 g; Rf.0.52 Bz:EtAC::3:2). Further elution with 10% ether gave the starting material (0.4 g; Rf.0.4).

m.p. = 128.5-129.5°

$(\alpha)_D^{26}$ = +82.7 (c,0.532;d,0.22)

IR (ν) = Fig. 33.

λ max = 242 mμ

$C_{25}H_{38}O_3$ requires C=77.72, H=9.85; found C=77.71, H=10.54

3-keto-chol-4-enic acid

Methyl 3-keto-chol-4-enate (1.5 g) was refluxed for eight hours with aqueous methanolic K_2CO_3 (1 g). Equal amount of water was added and the reaction mixture neutralized with acetic acid, extracted with ether, dried and evaporated. The residue was crystallized from ethanol.

m.p. = 186-188°

$(\alpha)_D^{26}$ = +83.56

λ max = 242 mμ

IR (ν) = Fig. 34.

$C_{24}H_{36}O_3$ requires C=77.42, H=9.67; found C=77.60, H=9.71

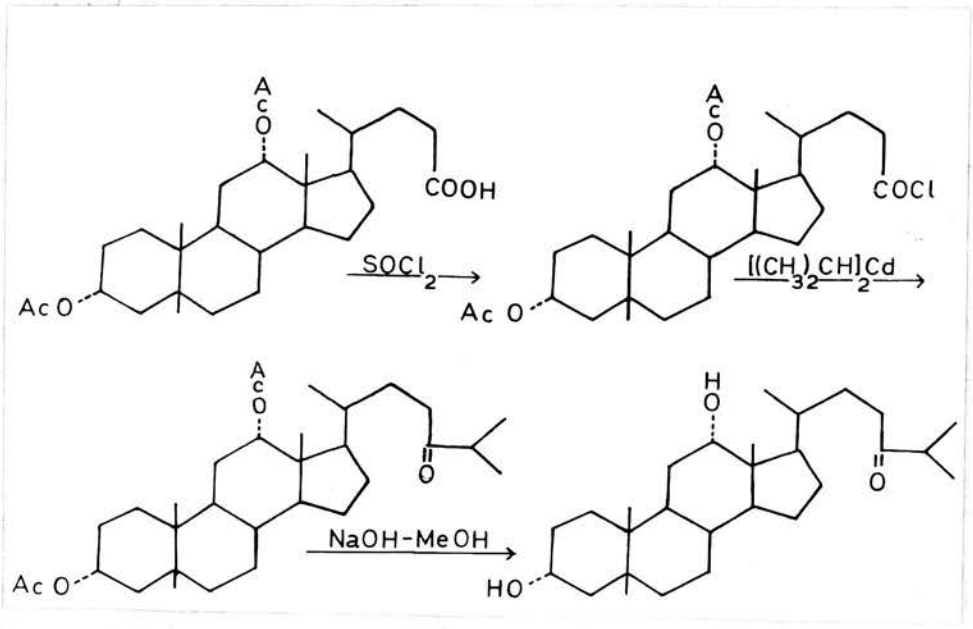

AcO
COOH
SOCl2
AcO
AcO
COCl
[(CH3)2CH]2Cd
AcO
AcO
O
NaOH-MeOH
AcO
HO
HO
O

Figure. 22.

5β-cholestan-3α,12α-diol-24-one

1. Deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid) was acetylated in the usual way.

The oily residue of 3α,12α-diacetoxy-5β-cholanic acid was dried under high vacuum giving rise to a dry sponge-like material which was treated with freshly distilled thionyl chloride in dry ether and worked up according to Cole and Julian (1945). The diacetoxy deoxycholyl chloride thus obtained was dissolved in dry benzene and the solution added to the di-isopropyl-cadmium prepared according to Cason and Prout (1955) using cadmium bromide instead of cadmium chloride. The reaction mixture was worked up after Cole and Julian (1945). The residue was hydrolysed with methanolic sodium hydroxide and extracted with ether. The residue from the ether extract was chromatographed on deactivated (5%) neutral alumina. A benzene solution was passed through the column and eluted with increasing amounts of ethyl acetate in benzene. Ethyl acetate in benzene (75%) eluted an oil which showed a single spot on TLC (Rf.0.22; benzene:ethyl acetate:acetone::10:5:3). The oily residue could not be crystallized but when the ether solution was evaporated under high vacuo, it gave rise to a dry powder, which was dried in a drying pistol at 80° to yield a pale yellow glass.

m.p. = 84-86°

$(\alpha)_D^{25}$ = +43.2

IR (ν) = Fig. 35.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C=76.82, H=11.14

Cholestan-3β,5α,6β-triol

This compound was prepared according to the method of Fieser and Rajagopalan, (1949).

Preparation. Cholesterol (20 g) was suspended in 88% formic acid (200 ml) and heated briefly to 70-80°. The 3β-formate thus formed separated as an oily layer, and the mixture was allowed to cool to room temperature. Hydrogen peroxide (20 ml of 30%) was added and the reaction mixture shaken occasionally for one hour and then left overnight. It was then treated with hot water (300 ml), shaken, cooled and the white solid filtered, dried, hydrolysed in aqueous methanolic sodium hydroxide and worked up as usual.

Chromatography. The white solid thus obtained was chromatographed on active alumina (Spence type '0'). The material was dissolved in acetone:ether::1:1 and the column eluted with increasing amounts of acetone whereupon methanol:acetone (1:3) eluted the triol. This fraction was crystallized from aqueous acetone and afforded white needles.

Physical characteristics:

m.p. = 231-233° Reported 237-239° and 229-233°

$[\alpha]_D^{26}$ = +3 (c,1.0;d,0.015)

IR (ν) = Fig. 36.

$C_{27}H_{48}O_3$ requires C=77.14, H=11.43; found C=74.66, H=10.96.

$C_{27}H_{48}O_3 \times H_2O$ requires C=74.24, H=10.95.

Cholestan-3β,5α-diol-6-one

This compound was prepared following the method of Fieser and Rajagopalan (1949[a]).

Preparation. Cholestane-3β,5α,6β-triol (.3 g) was dissolved in 10% aqueous dioxane (4 ml) and the solution treated with N-bromosuccinimide (0.2 g). The colour of the solution changed from yellow to light orange, light yellow and then colourless, whereupon the reaction product began to crystallize out. The mixture was kept at room temperature for ten minutes and then allowed to crystallize in the cold, filtered, and washed with 50% methanol. The mother liquor was diluted with water, extracted with ether, washed, dried and concentrated until crystallization started, and a second crop of crystals was obtained (m.p. 231-232° reported 232-233°). On TLC analysis the product showed a considerable amount of starting material. Purification was effected by chromatography on neutral alumina (10 g).

Chromatography. The material was dissolved in 40% ethyl acetate in benzene and adsorbed on the column prepared in benzene and eluted with increasing percentages of ethyl acetate in benzene followed by pure ethyl acetate then increasing amounts of acetone in ethyl acetate. Pure acetone and 5% methanol in acetone gave cholestane-3β,5α-diol-6-one. Crystallization from acetone-water afforded white needles (Rf.0.356 in benzene:ethyl acetate::7:13)

m.p. = 236–239° Reported 232–233°

$(\alpha)_D^{27}$ = -40.14 (c,0.548;d,0.11) -31.9 (Schultz, 1959)

IR (ν) = Fig. 37.

$C_{27}H_{46}O_3$ requires C=77.51, H=11.00; found C= 77.06, H= 11.41.

Cholestane-3β-ol-5α,6α-epoxide

Cholesterol benzoate (10 g) was dissolved in chloroform (80 ml) and a solution of monoperphthalic acid in ether (40 ml; 120 mg/ml) was added. The mixture was kept at 0° overnight and then at room temperature for twenty-four hours. The precipitated phthalic acid was filtered off and washed with more ether. The chloroform-ether solution was washed with sodium carbonate solution (5%) to remove excess peracid, and then washed neutral with water, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in boiling ethyl acetate. The first crop consisted of "α-cholesteryl benzoate" (m.p. 169-170° reported 168-169°). Cholestane-3β-ol-5α,6α-epoxide was obtained on hydrolysis of "α-cholesteryl benzoate" in methanolic potassium carbonate. The reaction mixture was refluxed for seven hours and poured into water, extracted with ether and worked up as usual. The residue on TLC showed incomplete hydrolysis and was chromatographed on alumina (Spence '0' type). The mixture was dissolved in benzene:ether::1:1 and applied on to the column and then eluted with increasing amounts of ether in benzene, followed by acetone-ether mixtures. "α-cholesteryl benzoate" eluted with 20% acetone in ether and cholestane-3β-ol-5α,6α-epoxide was eluted with 35% acetone in ether. The residue from this fraction was crystallized from methanol (Rf.0.52 in benzene:ethyl acetate::7:13).

m.p. = 144° Reported 142.5°

$[\alpha]_D^{27}$ = -44 -40

IR (ν) = Fig. 38.

$C_{27}H_{46}O_2$ requires C=80.6, H=11.45; found C=78.65, H=11.54.

$(C_{27}H_{46}O_2)_2CH_3OH$ requires C=78.9, H=11.48.

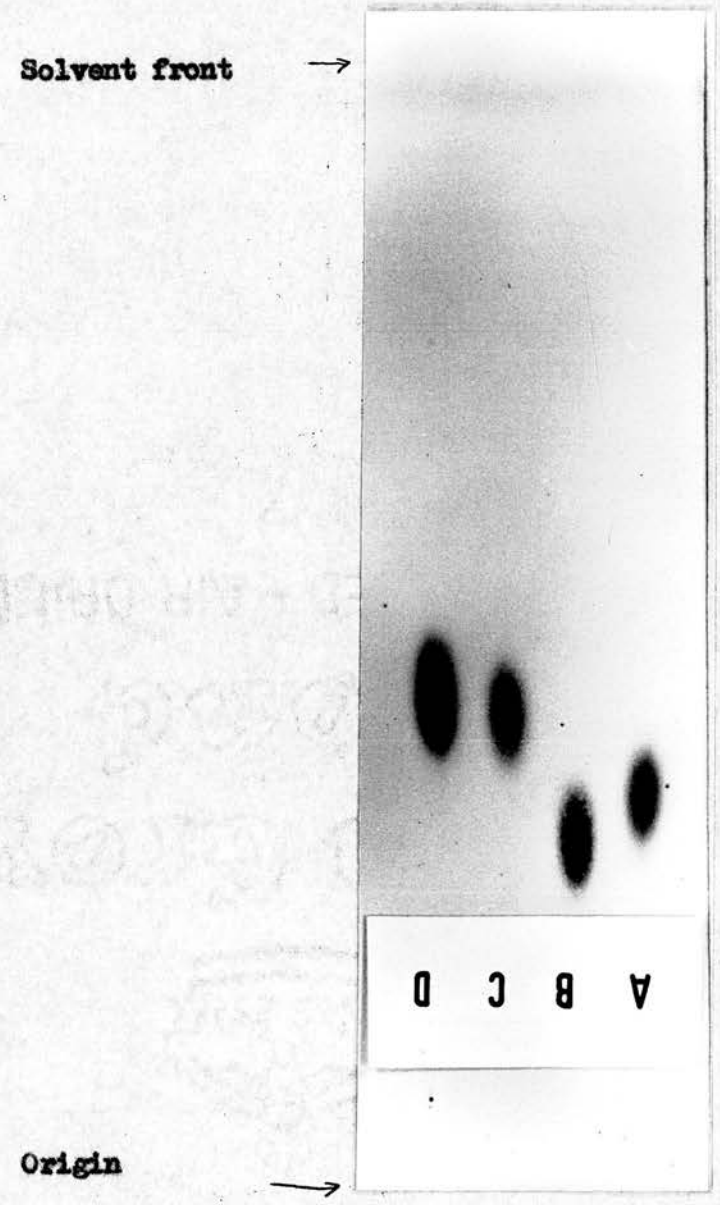
Cholest-6-en-3β-ol-5α-hydroperoxide

A solution of cholesterol (5 g) in anhydrous pyridine (100 ml) was photo-oxygenated for twenty-seven hours with haematoporphyrin hydrochloride (5 mg) using oxygen/5%CO_2. More photo-sensitiser (5 mg) was added after three and seven hours.

The mixture was taken to dryness in vacuo and the residue was treated with 1:1 mixture of glacial acetic acid and methanol (40 ml). The suspension was filtered and the residue was washed with cold methanol. Crystallization of the residue from hot aqueous methanol yielded white needles (2.8 g).

m.p. = 142° Reported 142°

$(\alpha)_D^{27}$ = +37.5°



Thin layer chromatogram showing relative mobilities of the following compounds.

(A) Cholest-5-en-3β,7β-diol.
(B) Cholestan-3β,5α-diol.
(C) Cholest-6-en-3β,5α-diol.
(D) Cholest-5-en-3β,4β-diol.

Solvent system :- benzene : ethyl acetate :: 1 : 2.

Cholestan-3β,5α-diol

Cholesterol (0.5 g) was dissolved in dry pyridine (40 ml) and haematoporphyrin hydrochloride (6 mg) was added. The solution was photo-oxygenated for twelve hours. Pyridine was evaporated in vacuo and the residue was crystallized from methanol several times. Colourless needles (m.p. 140°, $[\alpha]_D^{27}$ = +35) were dissolved in dry ether and reduced with hydrogen over 10% palladium on charcoal as catalyst. When the hydroperoxide was completely reduced there were two main spots on TLC, (Rf.0.33, light blue colour with phospho-tungstic acid; Rf.0.26, pink colour with PTA) corresponding to the cholest-6-en-3β,5α-diol and cholestane-3β,5α-diol respectively. There were also traces of 7α-hydroxycholesterol (Rf.0.23 dark blue colour with PTA). The reduction was continued until there was no blue spot on TLC corresponding to the more mobile cholest-6-en-3β,5α-diol (Rf.0.33). The catalyst was then filtered off, the ether evaporated, and the residue acetylated with acetic anhydride in pyridine at room temperature for twelve hours and worked up as usual. The ether extract was evaporated and the residue dissolved in benzene:petroleum ether::1:1 and chromatographed on a neutral alumina (15 g) column, prepared in petroleum ether. The column was eluted with benzene followed by 2% and 5% ethyl acetate in benzene which removed less polar impurities such as cholest-5-en,3β,7α-diacetate. Ethyl acetate in benzene (10% and 50% gave cholest-6-en-3β-acetoxy-5α-ol. The residue was

completely hydrolysed with 5% aqueous methanolic sodium hydroxide by warming and then leaving at room temperature for three hours.

Water was added and the reaction mixture left in the cold for crystallization. Silky needles separated and the mother liquor was extracted twice with ether. The crystals and ether extracts were combined, washed with water and dried. Crystallization from methanol afforded pure cholestan-3β,5∝-diol.

m.p. = 225° Reported 224°

$(\alpha)_D^{26}$ = +21.8 +21.2

IR (ν) = Fig. 39.

$C_{27}H_{48}O_2$ requires C=80.2, H=11.88; found C=80.33, H=11.91.

Cholest-6-en-3β,5α-diol

Cholest-6-en-3β-ol-5α-hydroperoxide (240 mg) prepared according to Schenck et. al (1957) was dissolved in tetrahydrofuran (10 ml) and reduced with lithium aluminium hydride (0.2 g) at reflux temperature for one hour. The reaction mixture was worked up as before and the residue chromatographed on neutral alumina (12 g). The reduction mixture was dissolved in benzene and chromatographed with increasing percentages of ethyl acetate in benzene. Each fraction (25 ml) of 5%, 10%, 15%, 20%, 25%, 30%, 40% and 50% ethyl acetate in benzene removed traces of more mobile impurities. In the same way 60%, 80%, 90% and then pure ethyl acetate gave a mixture of 5α- and 7α-diols (Rfs.0.33 and 0.23 in benzene:ethyl acetate::1:1). Pure cholest-6-en-3β,5α-diol (assessed by TLC) could not be obtained after several crystallizations from methanol. The mixture (195 mg) was acetylated at room temperature in pyridine for ten hours and cholest-6-en-3β-acetoxy-5α-ol (m.p. 145° reported 143°) was separated easily on column chromatography from cholest-5-en-3β,7α-diol-diacetate, as described on page 122 for cholestan-3β-acetoxy-5α-ol. Deactivated (1%) neutral alumina (12 g) was used as adsorbent and elution with 10% and 15% ethyl acetate in benzene gave pure cholest-6-en-3β-acetoxy-5α-ol (160 mg). Hydrolysis in 5% methanolic sodium hydroxide afforded cholest-6-en-3β,5α-diol. Recrystallization from methanol yielded white needles.

m.p. = 182.5° (with previous softening between 170-175°) Reported 181°

$(\alpha)_D^{26}$ = -18 (c,0564;d,0.05) -16

IR ( ) = Fig. 40.

$C_{27}H_{46}O_2$ requires C=80.59, H=11.4; found C=80.09, H=11.61

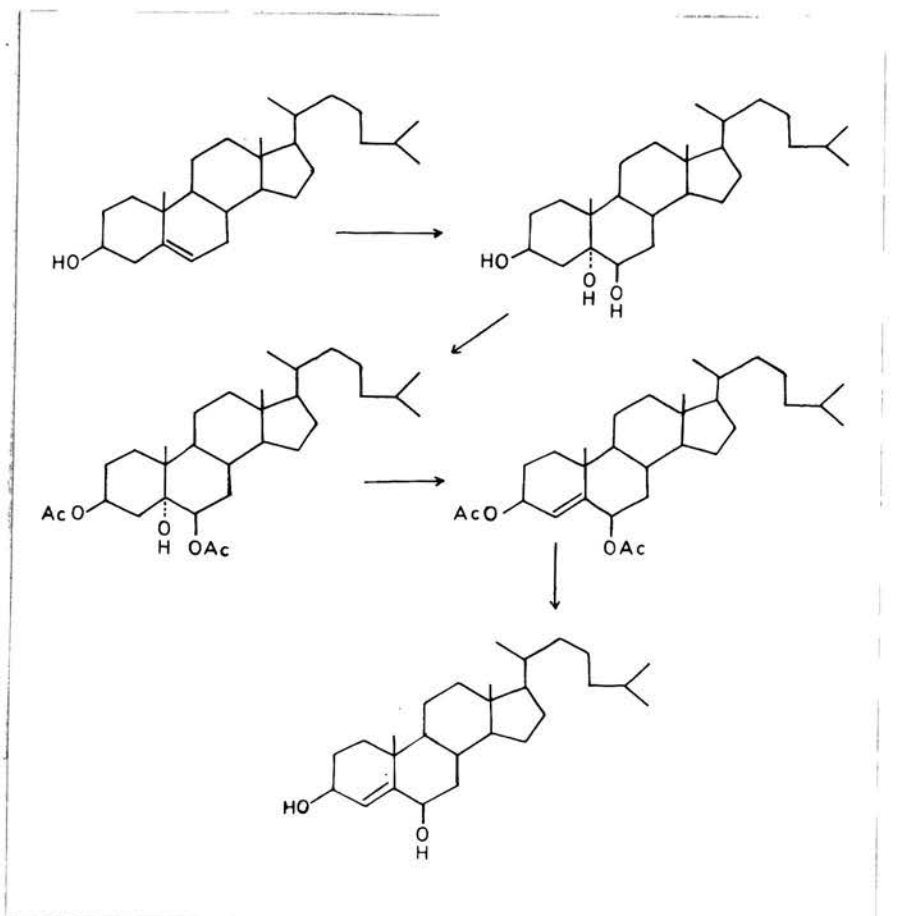


Figure. 23.

Cholest-4-en-3β-6β-diol

Cholestan-3β,5α,6β-triol-3β,6β-diacetate

Cholestan-3β,5α,6β-triol (700 mg) was dissolved in pyridine (2 ml) and acetic anhydride (2.2 ml). The solution was allowed to stand for twenty-four hours at 4°C. The reaction mixture was poured on to crushed ice, extracted with ether, washed with water, dried and evaporated. The residue (820 mg) was crystallized from methanol and white needles (760 mg) were obtained (Rf.0.24 benzene:petroleum ether:ether:: 2:2:1).

m.p. = 170-170.5 Reported 166°

$(\alpha)_D^{26}$ = -46.8(c,1.01;d,.235) -45

IR ( ) = Fig. 41.

$C_{31}H_{52}O_5$ requires C=74.00, H=10.31; found C=73.82, H=10.51.

Cholest-4-en-3β,6β-diol-diacetate

Cholestan-3β,6β-diacetoxy-5α-ol (0.58 g) was dissolved in anhydrous pyridine (3 ml) and the solution was cooled to 0°C. Thionyl chloride (0.2 ml) was added dropwise with shaking, the solution turned yellow and a white solid formed (Petrow, Rosenheim and Starling 1938). The mixture was kept at 0°C for fifteen minutes and then allowed to reach room temperature, water was added and the white solid turned oily. After some hours the oily mass solidified again, and was filtered, washed, dried, dissolved in petroleum ether, chromatographed on neutral alumina (15 g) and eluted with increasing amounts of benzene in

petroleum ether. Benzene in petroleum ether (80%) and then pure benzene eluted the least polar impurity. Ethyl acetate in benzene (4%, 6% and 12%) gave cholest-4-en-3β,6β-diacetate (360 mg). Crystallization from methanol afforded needles (Rf.0.68 benzene:petroleum ether:ether::1:1:1). Ethyl acetate in benzene (20%, 30% and 50%) gave 190 mg. of the starting material (Rf.0.3 in benzene:petroleum ether:ether:: 1:1:1).

m.p. = 135° Reported 136°

$(\alpha)_D^{26}$ = -13.2 (c,0.76;d,0.05) -13

IR (ν) = Fig. 42.

$C_{31}H_{50}O_4$ requires C=76.54, H=10.29; found C=76.09, H=10.51

Cholest-4-en-3β,6β-diol

The diacetate was hydrolysed in 5% methanolic sodium hydroxide overnight at room temperature, diluted with water and the precipitate filtered, washed and dried. Cholest-4-en-3β,6β-diol was obtained pure by TLC (Rf.0.2, benzene: ethyl acetate::1:1).

m.p. = 254-255° Reported 256-258°

IR (ν) = Fig. 43.

$C_{27}H_{46}O_2$ requires C=80.59, H=11.44; found C=81.00; H=11.8

DISCUSSION

DISCUSSION

In most biological hydroxylation whenever an oxygen atom is introduced at a carbon atom a direct replacement of the hydrogen atom with the hydroxyl group takes place and molecular oxygen is usually involved. In the cholesterol molecule many carbon atoms can be hydroxylated but the most important positions affected are 1, 2, 4, 5, 6, 7, 11, 12, 17, 20, 22, 24, 25 and 26. The monohydroxy derivatives of cholesterol synthesized (diols) include all these positions except 11 and 17.

In the production of triols (fig.18, IV a,b,c and d) having 3β, and 7α-hydroxyls and a 5,6-double bond with additional hydroxyl at position 12, 24, 25 or 26, advantage was taken of the method of photo-oxygenation originally developed by Schenck and further studied by Naqui and Boyd (1964). The conformation and position of the newly introduced group in the formation of triols from the diols was based on the following considerations.

Photo-oxygenation of cholesterol in pyridine is known to yield mainly cholest-6-en-3β-ol-5α-hydroperoxide, which isomerises to cholest-5-en-3β-ol-7α-hydroperoxide in chloroform solution. If such a chloroform solution of cholest-6-en-3β-ol-5α-hydroperoxide is reduced with $LiAlH_4$, and in case the isomerisation has not taken place quantitatively, then the reduction product will contain cholest-6-en-3β,5α-diol derived from the original 5α-hydroperoxide. The reduction product will also contain cholest-5-en-3β,7α-diol obtained from the isomerised 7α-hydroperoxide, and possibly cholest-5-en-3β,7β-diol

obtained from the cholest-5-en-3β-ol-7-one. The latter compound is a known product of decomposition of cholest-5-en-3β-ol-7α-hydroperoxide by dehydration. Since certain triols discussed in this work were also prepared through the photo-oxygenation of certain derivatives of cholesterol in pyridine, by analogy with cholesterol photo-oxygenation the newly introduced hydroxyl group may be either 5α or 7α or 7β. The extent of formation of these three hydroxyl groups will depend upon the extent of isomerization from the respective 5α-hydroperoxide to the 7α-hydroperoxide or their 7-oxo decomposition products.

If the rotation values of cholesterol (α_D -39°), cholest-5-en-3β,7α-diol (α_D -90°), cholest-5-en-3β,7β-diol (α_D +7) and cholest-6-en-3β,5α-diol (α_D -16) are considered then it will be observed that the introduction of a 7β-hydroxyl into the cholesterol molecule contributes a positive rotation of +97. Similarly the net change in the conversion of cholesterol to cholest-6-en-3β,5α-diol is responsible for a rotation contribution of +74°. Contrary to this the introduction of a 7α-hydroxyl group in cholesterol molecule contributes a negative rotation of -51°. If the cholest-5-en-3β,7α,12α-triol, cholest-5-en-3β,7α,24ξ-triol, cholest-5-en-3β,7α,25-triol and cholest-5-en-3β,7α,26-triol synthesized were the 7β- or the 5α-hydroxy derivatives, then their rotations would have been less negative than the starting diols as the contribution of the 7β-hydroxyl and 5α-hydroxyl groups to the molecular

DEOXYCHOLIC ACID.

↓

3α,12α-DIHYDROXYCOPROSTANE.

↓

5β-CHOLESTANE-3-ONE-12α-OL.

↓

CHOLEST-4-ENE-3-ONE-12α-OL.

↓

CHOLEST-4-ENE-3-ONE-12α-OL ENOL ACETATE.

↓

CHOLEST-5-ENE-3β,12α-DIOL.

Fig. 24.

rotation is positive. Since the contribution of the newly introduced group in the molecule accounts for the increment of negative rotation ranging from -46° to -49° it seems reasonable to assume that the newly introduced group was 7α-hydroxyl. This evidence in conjunction with other information led to the confirmation of the configuration of the hydroxyls introduced at C_7.

The 24-hydroxycholesterol, 25-hydroxycholesterol and 26-hydroxycholesterol were prepared by known methods. 12α-hydroxycholesterol was prepared according to the scheme given in fig. 24. Danielsson (1961) also synthesised this compound based on conventional α-bromination of a saturated 3-ketone of the 5β-series followed by dehydrobromination through a dinitrophenylhydrazone, and finally cleavage of the hydrazone with pyruvic acid. This procedure involved three steps and the yield was poor. We have converted the saturated 3-ketone to α,β-unsaturated ketone in one step using SeO_2 in ethanol in much better yields.

When these diols having an extra hydroxyl at position 12 or 24 or 25 or 26 with 5-ene-3β-ol moiety were photo-oxygenated in pyridine with a photosensitiser, mostly the 5α-hydroperoxide was formed. The isomerisation of the 5α-hydroperoxide derivative to the 7α-hydroperoxy compound was not smooth, (quantitative) as reported by Lythgoe and Trippett (1959) for cholest-5-en-3β-ol-5α-hydroperoxide. While this work was in progress Berseus, Danielsson, and Einarsson (1967) reported the

synthesis of cholest-5-en-3β,7α,12α-triol from cholest-5-en 3β-12α-diol diacetate. Their reaction sequence was based on the work of Bide et.al (1948) for the allylic 7-bromination by means of N-bromosuccinimide. Treatment of the 7-bromo derivative with Ag_2O according to Schaltegger and Mullner (1951) followed by reduction with $LiAlH_4$ gave the desired triol in about a 20% yield. In the present studies this triol was prepared directly from 12α-hydroxycholesterol by means of photo-oxygenation, which does not require any protection of the hydroxyl groups present in the molecule.

In the photo-oxygenation of cholesterol a high melting point compound (257-260°) was also isolated. Its I.R. spectrum showed hydroxyl peaks. On the basis of its solubility in alcohol and high melting point this was suspected to be cholest-4-en-3β,6β-diol. To establish its structure synthesis of cholest-4-en-3β,6β-diol was undertaken according to the scheme given in figure 23. Cholestan-3β,5α,6β-triol was prepared according to the method of Fieser and Rajagopalan (1949). The triol was acetylated in pyridine and acetic-anhydride at 4°C overnight. Under this condition the equatorial and therefore less hindered 3β, and 6β hydroxyl groups were acetylated and the tertiary 5α-hydroxy (axial) was left free.

In general axial alcohols are more readily dehydrated than their equatorial epimers if the hydroxyl and one of the hydrogen atoms on the adjacent carbon atoms are in the same

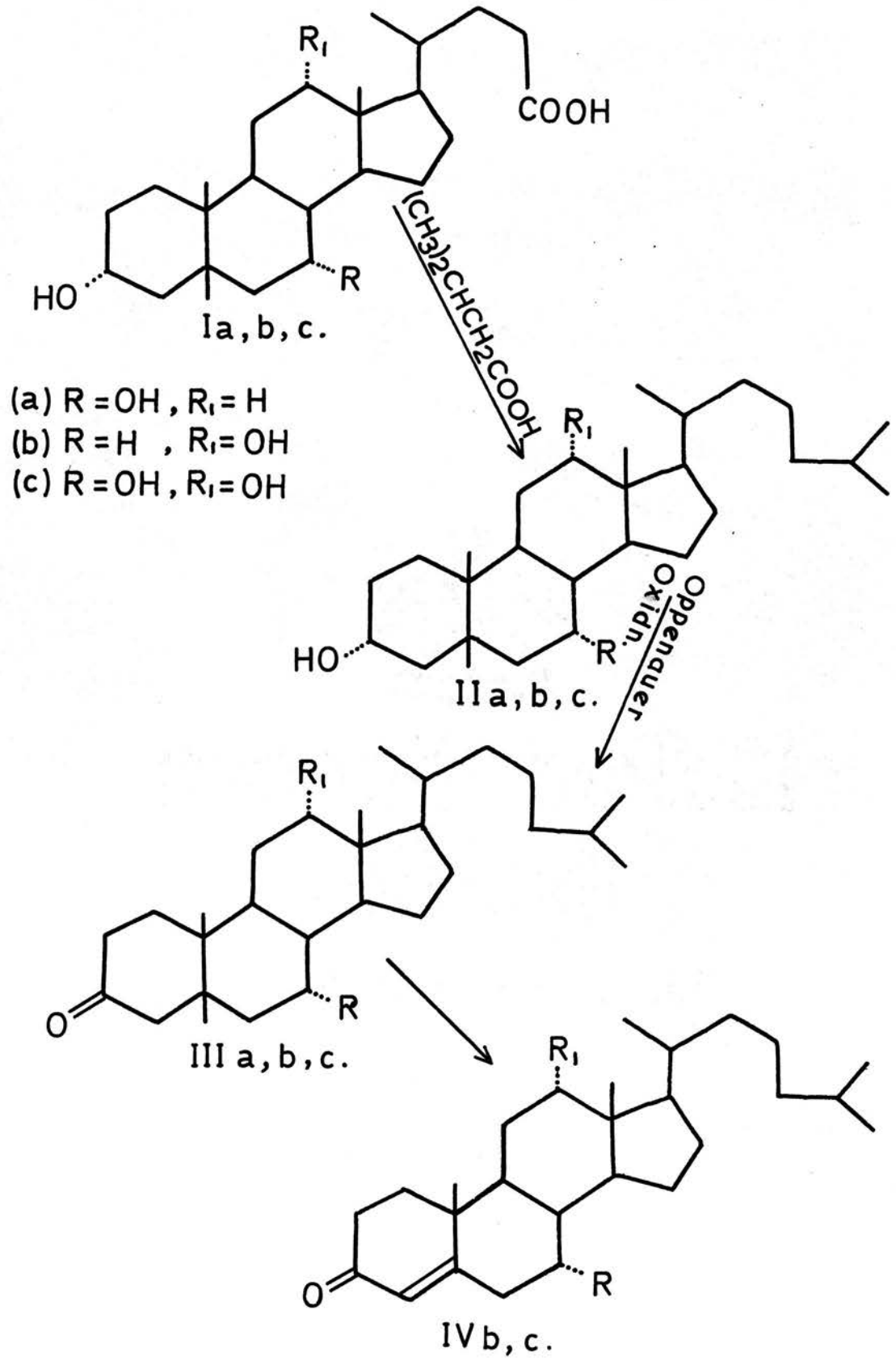

R1
COOH
HO
R
Ia, b, c.
(a) R = OH, R1 = H
(b) R = H , R1 = OH
(c) R = OH, R1 = OH
(CH3)2CHCH2COOH
R1
HO
R
IIa, b, c.
Oppenauer Oxidn.
R1
O
R
III a, b, c.
R1
O
R
IV b, c.

Fig. 25.

plane but trans in their conformation.

In cholestan-3β,6β-diacetoxy-5α-ol the tertiary 5α-hydroxyl (axial) was readily dehydrated in the 4 position by means of Darzen's reagent, because of the geometric relation of/4β-H: 5α-OH (trans: diaxial refer fig.2a.) which facilitates ready elimination of water. Hydrolysis at room temperature with alcoholic NaOH gave cholest-4-en-3β,6β-diol. A mixed melting point and co-chromatography showed that the compound obtained in photo-oxygenation was identical with the authentic cholest-4-en-3β,6β-diol.

An attempt to prepare cholest-4-en-3β,6β-diol from cholest-5-en-3β,4β-diol according to Petrow, Rosenheim and Starling (1943) by/a short treatment with acetic acid at reflux temperature, and then acetylation by means of acetic anhydride, resulted in cholest-5-en-3β,4β-diacetate.

Compounds having a 4-ene-3-one moiety with α-hydroxyl groups at positions 7 or 12,or both,were prepared according to the scheme given in figure 25. The preparation of various coprostanes (II) is based on the method of Kazuno and Mori (1954) as simplified by Bergstrom and Krabisch (1957) which involves electrolytic coupling of potassium salts of appropriate bile acids with potassium isovalerate. Since anodic coupling of the salts of the bile acids also occurs resulting in loss of bile acids as starting material, the use of excess of isovaleric acid was found to be advantageous in improving the yield of the desired product. Using isovaleric acid 10-12

times in excess 40-45% yields of these coprostanes were obtained.

The oxidation of a 3α-hydroxy group in the 5β(H) series to a ketone in the presence of a 7α-hydroxyl group or a 12α-hydroxyl group (or both) in these coprostanes cannot be achieved by such oxidizing agents as N-bromosuccinimide, sodium chromate or dichromate or chromium anhydride where the rate determining step is an attack on the less hindered equatorial C-H bond rather than O-H bond.

In the coprostane series, an α-hydroxyl group at position 3 is equatorial with respect to the ring A therefore less hindered, hence the hydrogen is axial and hindered. Such a conformation of the α-hydroxyl group at position 3 makes acetylation easy but oxidation more difficult. The situation is reversed in the case of the axial hydroxyls at positions 7α and 12α which have easily accessible hydrogens in the least hindered equatorial conformation. Further the 12α-hydroxyl induces an instability factor in the ring C by a 1:3 interaction with the α-hydrogens at positions 9, 14 and 17. Similarly the α-hydroxyl at C_7 also exhibits 1:3 interactions with the α-hydrogens at 9 and 14 coupled with an equivalent 1:3 interaction with the methylene group at C_4 (fig.2b). Since the transformation of a hydroxylic tetrahedral C-atom to a trigonal carbonyl carbon atom eliminates some or all of these repulsive interactions responsible for such instability, a relief of these strains provides a driving force sufficient

to promote oxidation of the axial hydroxyl groups (Schreiber and Eschenmoser 1955). Because of these interactions coupled with the equatorial orientation of the hydrogen at C_7 or C_{12} (or both) the α-hydroxyl groups at these carbon atoms will be oxidized readily in the presence of the 3α-hydroxyl by the oxidizing agents mentioned above.

In the Oppenauer oxidation, although oxidation at C_3 of an equatorial hydroxyl may be facilitated by a 5,6-ethenoid linkage, the presence of a homoallylic double bond does not appear to be essential, as oxidation of the saturated compounds of the 5β-series having 7α and 12α-hydroxyls, for example the bile acids or their methyl esters proceeds smoothly and remains confined to the 3 position (Riegel and McIntosh 1944). This points to the fact that in the case of the Oppenauer oxidation the rate of oxidation is dependent on the ease of severance of the O-H bond and therefore will depend upon the degree of hindrance of the hydroxyl groups. The attack by aluminium alkoxides is so selective (or stereospecific) that the α-hydroxyls at C_7 or C_{12} or both in the coprostane series need not be protected. The 12α-hydroxyl group was found to be least reactive, the 7α-hydroxyl group intermediate and the 3α-hydroxyl group was most reactive. Further it was found that the 7α-hydroxyl group was quite reactive in the absence of a 12α-hydroxy group in the same molecule, but in the presence of a 12α-hydroxyl group the reactivity of the 7α-hydroxyl group was considerable reduced. This conclusion is

based on the fact that when 5β-cholestan-3α,7α-diol was oxidized by the Oppenauer method and the reaction was conducted for more than three to four hours lower yields of 5β-cholestan-3-one-7α-ol were obtained due to the formation of a dione (see Danielsson 1961 also) while the oxidation of 3α,7α,12α-trihydroxy coprostane and 3α,12α-dihydroxycoprostane was conducted for thirteen hours with only 15% and 4% conversion to other products was found calculated on the basis of solid residues obtained from different chromatographic fractions.

An allylic double bond is usually introduced in steroids with a carbonyl by bromination to the α-bromo ketone which on dehydrobromination would result in an α,β-unsaturated ketone. This reaction depends on the direction of enolization which appears to be controlled by the stereochemical configuration of the nearest bridge-head. For example the 3-ketones of the 5α-series enolize to give 2-enes, whereas a 3-ketone of the 5β-series is converted preferentially to the Δ^3-enol acetate (Dauben, Micheli and Eastham 1952). Consequently bromination of a 3-ketone of the allo-series gives a 2-bromoketone and a 3-ketone of the normal series affords a 4-bromoketone. Since the purpose of this synthesis was to obtain a 4-ene-3-one moiety, the 3-ketones of the 5β-series were employed. Bromination with bromine or N-bromosuccinimide affords both axial and equatorial epimers. In the 5β-series the 4β-bromo is the stable configuration and is thus not susceptible to a transelimination of HBr.

Rosenkranz, Mancera, Gatica and Djerassi (1950) have described a novel method of introducing a 4-ene-3-one moiety in the 3 keto-allosteroids, which involves the formation of 2α,4α-dibromoketone. Brief treatment of this dibromoketone with sodium iodide in acetone solution gives a 2ξ iodo-4α-bromoketone, which on boiling with collidine undergoes deiodination and dehydrobromination, yielding a 4-ene-3-one moiety. The conversion of 2α,4α-dibromo-allo-3-ketone to 4-ene-3-one can be accomplished in 60% overall yield without isolation of the intermediates.

2β,4β-dibromo-3-ketones of the 5β-series can be obtained in 90% yield (Djerassi and Rosenkranz 1951) but unfortunately these compounds do not react with sodium iodide in acetone.

The monobromo-3-ketones of the 5β-series dehydrobrominate in poor yield when refluxed with pyridine because of the 2 isomeric, 4-bromo derivatives. Facile transelimination of HBr only occurs with the α-stereoisomeric 4-bromo compounds.

However if the 3-keto, 4-bromo-derivatives were treated with a slight excess of dinitrophenyl hydrazine with or without the addition of sodium acetate in the absence of oxygen, 4-bromo dinitrophenylhydrazones were formed rapidly which dehydrobrominated to 4-ene-3-keto-hydrazones in 90% yield. Furthermore cleavage by exchange with pyruvic acid in the presence of HBr regenerated the α,β-unsaturated ketone almost quantitatively (Mattox and Kendall 1948). The practical value of this method totally depends upon the quantitative

regeneration of the unsaturated ketone from its hydrazone. It is believed that the water content of the pyruvic acid greatly influences the extent of cleavage, and therefore the results are dependent on the purity of the pyruvic acid. Djerassi (1949) has modified the method of Mattox and Kendell for pyruvic acid cleavage of hydrazones, and has claimed to obtain reproducible results. However in our hands Djerassi's method has also given only partial regeneration of unsaturated ketone. Our yield corresponds with that reported by Danielsson (1961).

Further, this method of introducing a double bond through α-bromoketone and subsequent dehydrobromination can be applied only in those steroids where no 7α-hydroxy function is present. The presence of 4,5-double bond activates the C_6-hydrogen atoms and the elimination of water from the 6β-hydrogen (axial) and 7α-hydroxyl (axial) group gives a dienone under the influence of acid or alkali. The 7α-hydroxy group of cholest-4-en-3-one-7α-ol and cholest-4-en-3-one-7α,12α-diol becomes so acid and alkali labile that even an acetoxy group is eliminated when boiled with pyridine or p-toluene sulphonic acid in a neutral solvent.

A search in the literature revealed that 2,3-dichloro-5,6 dicyano-benzoquinone (DDQ) mediated dehydrogenation can be used for the introduction of a single double bond in a saturated 3-ketone, and the structure of the starting material determines which hydrogen atoms are removed.

Ringold and Turner (1962) reported that the DDQ dehydrogenation of 5α-androstane-17β-ol-3-one and 5β-androstane-17β-ol-3-one in boiling xylene follows the known direction of enolization leading to 1-ene-3-one and 4-ene-3-one respectively, as the major product, but the reaction time was not given. An exploratory experiment was conducted refluxing 100 mg of coprostan-3-one-12α-ol, 1:1 equivalent of DDQ in 10 ml of dioxane and the reaction followed on TLC using a solvent system that separated the starting material, cholest-4-en-3-one-12α-ol and cholest-1,4-diene-3-one-12α-ol from each other. After every hour the reaction mixture was run on TLC and the amounts of the products compared. During the first four hours the amount of cholest-4-en-3-one-12α-ol increased, and then the quantity decreased as cholest-1,4-diene-3-one-12α-ol formed later, and in eight hours the reaction mixture seemed to have acquired equilibrium. Although the reaction was carried out for fifteen hours no change in the ratio of the products was noted. No doubt cholest-4-en-3-one-12α-ol was the major product, but still a large amount of starting material was present; it was therefore concluded that this method may be of theoretical interest, but it cannot be used for this particular preparative purpose. (See Walker and Hiebert 1967 for a review on DDQ).

Selenium dioxide has also been used for dehydrogenation purpose. By analogy with bromination of a 3-ketone (5β-) series, this reagent would be expected to attack first the

4,5-position. This compound when used at reflux temperature with acetic acid or t-butanol with or without acetic acid is excellent for converting 4-ene-3-ones or 3-ketones to 1,4-diene-3-ones. When used in 96% ethanol at a temperature below its boiling point, SeO_2 has given yields of 4-ene-3-one compounds from saturated 3-ketones of the 5β-series, and further dehydrogenation at the 1,2-position was very much arrested. At reflux temperature the yield of the 4-ene-3-one derivative was considerably reduced because at higher temperatures or in the presence of acetic acid, formation of 1,4-diene-3-ones from 4-ene-3-ones was facilitated. There seemed to be a difference in the rate of dehydrogenation of 5β-cholestan-3-one-7α-ol, 5β-cholestan-3-one-7α,12α-diol and 5β-cholestan-3-one-12α-ol with SeO_2, which suggested that a 7α-hydroxyl group had a promoting effect on the 4,5-dehydrogenation, contrary to the expectation that its presence might hinder the α-approach of the reagent.

When several reagents perform similar functions the choice falls to that reagent which gives not only a better yield but also can be used on a variety of compounds. In this work the best results were obtained by converting the 3-ketones of the 5β-series to 4-ene-3-one derivatives, even when the 7α-hydroxy function was present in the molecule, by the use of SeO_2 dehydrogenation in ethanol.

12α-hydroxycholesterol was prepared through the enol acetate of cholest-4-en-3-one-12α-ol which on reduction with

$NaBH_4$ in aqueous methanol according to the method of Balleau and Gallagher (1951) for the preparation of cholesterol from cholest-4-en-3-one, furnished 12α-hydroxycholesterol in good yield. Preliminary experiments with cholest-4-en-3-one showed that the proportion of cholesterol formed was favoured by lower/a temperature but no improvement in the yield of cholesterol was found when "inverse addition" technique was used contrary to the finding of Dauben and Eastham (1951). Since small amounts of 4-ene-3α- and 3β-alcohols were also expected to be formed, the reduction mixture was refluxed with acidified methanol, and/the allylic alcohols were dehydrated to 3,5-dienes through the intermediate formation of 2,4-dienes. The 3β-and 3α-hydroxyl groups with a 5,6-double bond were unaffected, and the 12α-hydroxyl was not dehydrated. The 3β-conformation of/the hydroxy group was confirmed by its formation of an insoluble digitonide complex, and the 5-ene-3β-ol grouping ascertained by its colour response with 50% H_2SO_4 (v/v). The presence of a 5,6-double bond is also indicated from the consideration of molecular rotation relationship. A comparison of specific rotations of cholest-5-en-3α-ol (α_D-45) and 5β-cholestane-3α-ol (α_D+30) shows a negative rotational contribution (-75°) for the 5,6-double bond. A contribution of -69.3° is observed when the rotations of 5β-cholestane-3α,12α-diol (α_D+41.3°) and cholest-5-en-3β,12α-diol (α_D-28°) are compared.

As a rule, hydride reduction of a non-hindered ketone

gives predominantly the equatorial isomer, and the hindered ketones give axial alcohols. The proportion of 3β-isomer formed suggests that the reduction of the enol acetate after hydrolysis has taken place through the formation of a ketone before the isolated double bond moved to the stable allylic position.

This method of converting 4-ene-3-ones to 5-ene-3β-ols could not be applied for the preparation of cholest-5-en-3β, 7α,12α-triol because in the preparation of/the enol acetate of the 4-ene-3-one moiety/this involved acid catalysed conditions to which the 7α-hydroxy function in the starting material was labile. Although very mild conditions for the enolization were used (Djerassi et. al 1952) cholest-4,6-diene-12α-ol-3-one was rapidly formed (λmax 282).

Cholest-4-en-3-one-7α-ol was prepared according to the sequence given in figure 19.

Nickon and Bagli (1961) prepared the dienone III directly from cholesterol using the Wettstein modification of the Oppenaur oxidation, which proceeds via a cyclic intermediate involving the 7α-hydrogen, and they reported yields up to 62%.

The dienone III was obtained in/a two step synthesis. Cholesterol was converted to cholest-4-en-3-one using the original Oppenauer oxidation. The conversion of cholest-4-en-3-one to dienone by a chloranil-mediated dehydrogenation was based on the work of Agnello and Laubach (1960) for the dehydrogenation of corticosterone with chloranil. In 1957

these workers reported that the steroidal 4-ene-3-ones were converted to the corresponding 4,6-diene-3-one by the action of chloranil in solvents such as boiling t-butanol, or xylene and suggested that the reaction involved slow enolization to a 3,5-diene-3-ol followed by a quinone abstraction of a hydride ion from C_7. We conducted this reaction in t-butanol and found it to be complete in three hours, without using any catalyst such as p-toluene sulphonic acid for promoting enolization. The work up procedure was modified and 65-70% of crystalline dienone was obtained, and the compound characterized by its absorption maximum at 282 mμ etc.

Compound IV was prepared by the method of Nickon and Bagli (1961). The olefine-peracid reaction has been used for epoxidation in steroids. The electrophilic character of the reagent attacking the double bond may be inferred from the fact that electron attracting substituents such as carbonyl, carboxyl etc. near the unsaturated bond reduced the reaction velocity in accordance with their inductive properties whereas the electron releasing substituents e.g. alkyl groups accelerate the rate of epoxidation (Swern 1949).

The bulky angular methyl groups on the front (β) side of the molecule cause most reagents to approach from the rear (α) side of the molecule (Fieser 1950). In this connection peracid reactions are particularly stereospecific and α-epoxides are usually obtained from olefines if (a) the ring system is relatively flat with 5α, 8β,9α, 10β,14α, configuration,

(A/B trans) and (b) additional bulky groups of α-configuration are not too close to the double bond. However an exception to this postulation is the behaviour of the α,β-unsaturated alcohols which seem to exert a promoting effect in the formation of epoxides together with a directive influence to give cis-epoxy-alcohols. This behaviour of the 7β-hydroxyl is of interest because the shielding effect of the angular methyl groups on the β-face is pronounced. Nevertheless the predominant formation of a β-epoxide from cholest-5-en-3β-benzoyloxy-7β-ol shows that the frontal approach of the reagent is facilitated by the hydroxyl group and is more favoured than the approach from the less hindered rear side of the molecule. This behaviour can be explained by assuming that hydrogen bonding causes an association of the reactant favourable for interaction between the electrophilic peracid oxygen and the double bond. We believe that the formation of the 5β,6β-epoxide when cholesterol is epoxidized with peracids is the result of such an interaction, although the effect of the 3β-hydroxyl will be less pronounced because of its homoallylic nature with respect to the double bond at the 5,6-position.

The attack of monoperphthalic acid on the dienone was stereospecifically on the 6,7-double bond from the α-side of the molecule, and exclusively the 6α,7α-epoxide was formed, as shown by its reduction product which was exclusively the 7α-hydroxy isomer. In the reduction of a steroidal epoxide by means of $LiAlH_4$ we propose that the C-O bond was cleaved which was

maximally displaced from its normal position and therefore under greater strain. Hence the hydroxyl group formed would retain its normal configuration whereas the newly introduced hydrogen at C_6 would acquire the inverted β-conformation of the reduced ring B.

Re-oxidation of the isomeric allylic alcohols (V) was performed by MnO_2 prepared according to Mancera et.al (1953) but complete conversion to an α,β-unsaturated ketone was never achieved. Probably the α-isomer (axial) formed in the reduction was little oxidized whereas the β-isomer (equatorial) was oxidized in good yield.

Similar observation has been reported by Nickon and Bagli (1961) for different rates of oxidation of 5-ene-7α-ol and 5-ene-7β-ol. The quasi-equatorial 7β-isomer was oxidized ten times faster than the quasi-axial 7α-hydroxy compound.

3β-hydroxycholestenoic acid was prepared by a modification of the method of Dean and Whitehouse (1966) as given in fig. 16.

26-ketonorcholesterol acetate was reduced with $NaBH_4$ which does not reduce the acetate grouping (Cf $LiAlH_4$). The 26-hydroxynorcholesterol acetate thus obtained was converted into 26-chloro-norcholesterol acetate using freshly distilled thionyl chloride. Dean and Whitehouse (1966) used the bromocompound which they prepared from thionyl bromide prepared in turn from thionyl chloride. The chloro-compound was efficiently converted to the 26-cyanonorcholesterol acetate using KCN in DMS. This reaction was almost quantitative but was

accompanied by some hydrolysis of the 3β-acetate group. The cyano-compound was not purified but was hydrolysed by means of KOH in aqueous dioxane (10%), because when the hydrolysis was performed in alcoholic KOH according to Dean and Whitehouse (1966), even after twenty-four hours refluxing, most of the 26-cyano-compound was recovered from the alkaline hydrolysate. The hydrolysis was complete in aqueous dioxane at reflux temperature in six to seven hours. The position of the 26-carboxylic group was ascertained by its reduction to 26-hydroxycholesterol with excess of $LiAlH_4$ in THF, followed by comparison of the 26-hydroxycholesterol with an authentic sample.

The unknown compound isolated from the chromatography of 26-ketonorcholesterol acetate was identified as 3β-acetoxy-20-hydroxy-chol-5-enic acid lactone on the following basis. The I.R. spectrum showed acetate (3β) peak at 1738 cm^{-1} another peak at 1763 cm^{-1} characteristic of a 5-membered lactone. The melting point and optical rotation were almost identical to the known compound reported in the literature.

Chromatography of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol and their diacetates both on TLC on silica gel and neutral alumina column chromatography with or without water presented interesting problems. The relative mobilities of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol on partition chromatographic systems seem to conform to the generalization that the axial epimer is more mobile

(Savard 1954) than its equatorial epimer. Thus Schubert, Rose and Burger (1961) using paper partition chromatographic system and Mosbach, Nierenberg and Kendall (1953) using partition column chromatography found that the 7α-epimer was more mobile (polar) than the 7β-epimer.

Using adsorption chromatographic procedures, the reverse order of mobility was observed for the 7-epimeric hydroxy cholesterols, both on TLC (Claude and Beaumont 1966; Smith, Mathews, Bachmann and Reynolds 1966; Hutton and Boyd 1966) and column separations (Ruzicka, Prelog and Tagmann 1944).

Cholest-5-en-3β,7β-diol was more mobile on TLC silicic acid than the 7α-isomer, but cholest-5-en-3β-7β-diacetate and cholest-5-en-3β,7α-diacetate did not separate from each other in mixtures of benzene, and ethyl acetate or petroleum ether, diethyl ether and benzene. Further, on column chromatography using neutral alumina, little separation of cholest-5-en-3β,7β-diol and cholest-5-en-3β,7α-diol was observed using increasing amounts of acetone in petroleum ether. The addition of water to the adsorbent achieved the separation of the two 7-isomers. The 7β-isomer was more mobile than the 7α-isomer similar to their behaviour on silicic acid TLC. A mixture of cholest-5-en-3β,7β-diacetate and cholest-5-en-3β,7α-diacetate was resolved on neutral alumina with or without water, using mixtures of benzene and ethyl acetate for elution. Unexpectedly cholest-5-en-3β,7α-diacetate was found to be more mobile than cholest-5-en-3β,7β-diacetate.

Wintersteiner and Ruigh (1942) prepared cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol from the Meerwein Ponndorf reduction of cholest-5-en-3β-acetoxy-7-one. Fieser, Fieser and Chakravarti (1949) obtained the 7-isomeric diols on reduction of cholest-5-en-3β-acetoxy-7-one with $LiAlH_4$, benzoylation, and separation of 7β- and 7α-isomers by chromatography on alumina using petroleum ether and benzene mixtures, as described by Buser (1947).

In this work separation of cholest-5-en-3β,7α-diol and cholest-5-en-3β,7β-diol from a mixture was achieved after acetylation and chromatography on neutral alumina using petroleum ether-acetone mixtures, as well as chromatography of the free diols on 1% deactivated neutral alumina.

Cholest-5-en-3β-acetoxy-22-one was prepared as described by Fieser and Huang (1953). Reduction with $NaBH_4$ furnished cholest-5-en-3β-acetoxy-22ξ-ol.

On hydrolysis this compound gave cholest-5-en-3β,22ξ-diol, and treatment with phosphorous oxychloride gave the novel cholest-5,20(22)-diene-3β-ol, which on controlled epoxidation may give another novel cholest-5-en-3β-ol-20,22-epoxide of interest in cholesterol side chain cleavage reactions.

APPENDIX I

COLUMN AND TLC METHODS USED

Silicic acid is an excellent material for lipid separations including sterols. However the control of variables such as activation of silicic acid batches, water content, loading factor, particle size etc. for reproducibility of resolution prove difficult. For these reasons silicic acid was only used in cases where better separation on alumina could not be achieved. Similarly active alumina (basic) was seldom employed because this adsorbent is known to induce various reactions in steroids. For example when steroids with a 4-ene-3-one-7α-ol grouping were subjected to column chromatography on active alumina (basic) or neutral alumina in both cases dehydration of the 7α-hydroxyl group was encountered, giving rise to a dienone grouping. However these steroids were successfully chromatographed on deactivated neutral alumina. When acetates were chromatographed on active or neutral alumina, substantial hydrolysis was observed during the chromatography. This problem could be overcome by the addition of water to the neutral alumina.

Neutral alumina was prepared from active alumina (type 0 of Peter Spence) by the addition of dilute hydrochloric acid to a slurry of active alumina in methanol until the pH was slightly acidic. The slurry was stirred well and the excess acid neutralized by the addition of a further amount of active alumina. This treatment saved prolonged washing with water,

the methanol was decanted off, the alumina washed thrice with water, dried at 110 C for 12 hours and stored in an air tight container.

Deactivated grades of neutral alumina were prepared from neutral alumina on adding fixed amounts of water (1-10% v/w) and mixing thoroughly.

Silicic acid for column chromatography was prepared according to Ahrens and Hirsch (1958). Silicic acid was washed twice with methanol and after standing for half an hour the methanol was decanted off and it was washed twice with ether, dried in air and then activated for 12 hours at 110 C.

THIN LAYER CHROMATOGRAPHY

TLC has furnished a new approach for rapid and excellent resolution of closely related steroids in small amounts. The importance of this method is enhanced, since chromatography can be used for both qualitative and quantitative purposes. In addition to this it can also be utilized for preparative work where purification could not be achieved by ordinary column chromatography and other techniques of purification. New advances in this field have utilised the incorporation of $AgNO_3$ Barrett, Dallas and F.B. Padley (1962) or ammonical silver nitrate, Wood and Snyder (1966) into the silica gel or alumina TLC plates Kammereck, Lee, Paliokas and Schroepfer (1967) for the separation of steroids differing in the number of double bonds per molecule. The undecan-impregnated silica gel TLC system can be used for ordinary partition chromatography or reversed phase partition chromatography for the separation of sterols which differ only by an alkyl group or by the position of the double bond (Heftmann 1965).

TLC with the use of nondestructive reagents for the location of steroids is even more useful. Iodine either in vapour form or in solution is a useful nondestructive reagent which gives yellow stains. However no rigorous tests were done to this end. Even spraying the plate with water and viewing the chromatoplate against a dark background makes some spots visible. Ultra-violet absorbing steroids are

readily detected nondestructively as dark spots against fluorescent background by the inclusion of an inorganic phosphor such as zinc silicate or sulphide into the silicic acid. If the chromatogram is viewed under ultra-violet light and if the compound does not absorb ultra-violet radiation then the extinction is low and the difference in intensity between the zone and the background may be too small to be detected. However, if the compound adsorbed has an absorption maximum in the ultra-violet region then it will appear as a black spot on a fluorescent background. By using a series of interference filters in the range of the entire ultra-violet spectrum with a hydrogen lamp, this method will not only show the position of the substance on the plate, but, by determining the wave length of maximum extinction, would indicate the ultra-violet absorbing properties and thus information on its possible molecular structure can be obtained (Boyd and Hutton 1963).

TLC is used in kinetic or reaction rate determinations in synthetic organic chemistry and in the steroidal field. By using labelled materials coupled with radioautography or scintillation counting this method has proved very useful. Recent advances in kinetic studies of biological reactions are also based on "tracer" techniques coupled with TLC. The use of labelled substrates in synthetic organic work will have the added advantage over the use of "cold" materials

because the origin of the bye-product can be determined. It is possible to manipulate the side product and at the same time some idea about the nature of the intermediates involved in a reaction can be obtained if serial sampling and analysis is available. In steroidal reactions where the starting material or the products do not give a colour reaction, the isotopic labelling of the substrate can be used for identification and quantitation. Also because of its high sensitivity and accuracy information can be obtained on small amounts, where it may be almost impossible to detect a compound by a colour reaction.

Since the purpose of this work was mainly to synthesise possible intermediates in the degradation of cholesterol to bile acids, no effort was made to quantitate this method accurately either by colorimetry, dilution to minimum detectable amount or using labelled starting materials, in order to study the kinetics of the reaction. Visual comparisons were sufficient in order to evaluate the practicability of certain reactions. In this work TLC was used as a tool for the following purposes.

(a) To follow up the time-course of the reaction.

By running TLC plates of small aliquots of reaction mixtures at suitable intervals in various solvent systems the progress of the reaction can be followed. It can be noted whether the reaction was complete or had attained chemical equilibrium.

(b) To determine the purity of the product.

TLC was used as a tool for criteria of purity. Compounds prepared by the known methods although having the same physical and chemical constants as reported in the literature were often found to be heterogeneous on TLC. Only those compounds were regarded as pure which were homogeneous on TLC.

(c) To monitor the efficiency of chromatographic columns in determining the best solvent mixtures on pilot experiments for optimum resolution as well as to check different fractions for the desired product.

The conventional method for judging the performance of a chromatographic column was based on evaporation of each fraction for obtaining solid residue and analysing the individual residues for the desired product by crystallization etc. By using TLC a quick appraisal of the nature of the product in different fractions can be obtained, and therefore the desired product can be easily located and fractions which contain this product may be pooled.

(d) To evaluate the practicability of a chemical reaction on a small scale.

Such reactions were done on the milligram scale and the course of the reaction followed on TLC. With the help of this method it was possible to determine the extent of the desired product formed without using the cumbersome method of isolation of the pure product by crystallization.

(e) It was also used as a guide for the purification technique to be adopted. For example if the impurities on TLC were less mobile (colloquially - less polar) than the desired product and were well separated on TLC, purification was achieved by simple crystallization with a less polar solvent. When impurities were not very well separated from the desired product, column chromatography was used. It was not possible to transfer information obtained on TLC directly on to the column chromatography, particularly when separation of the isomeric products (α- and β-hydroxy isomers) was desired. It was often observed that although such isomers were separated on TLC, they could not be separated on column chromatography. On the other hand sometimes the acetates of such isomers could not be separated on TLC but were successfully separated on alumina column chromatography. Loev and Snader (1965) have described a method called "Dry-Column" chromatography, which gives the same resolution of components as is obtained on TLC. The method recommends the use of 3% silicic acid to give an activity comparable to silica gel TLC plates and to be packed in the column dry. The material is loaded by conventional methods and the column is developed by using the same solvent system which has been tried on TLC. The solvent mixture is allowed to move down the column by capillary action, not by liquid thrust, by controlling the solvent head to a maximum of 2 cms. and the ratio of sample to the adsorbent at 1:500. By the use of

certain specific colour reactions in conjunction with fluorescent TLC information is obtained about the structure of possible side products as well as the desired product. Colour reaction with co-chromatography or relative mobility versus reference steroids can be used very successfully in the identification and characterization of a product.

The "stationary phase" or adsorbent for TLC was prepared by spreading over a glass plate a slurry of 5% zinc silicate in kiesel gel G or H in water to give a uniform thickness (0.5 mm.). The plate, covered completely with the layer of silicic acid was left at room temperature for a few minutes until air-dried, and then activated at 110° for 30 minutes before use.

The substance in solution was applied to the plate and allowed to dry. The plate was then developed for 30–40 minutes in a suitable solvent system in a tank lined with paper for vapour saturation purposes. The plate was removed from the tank and after drying in the air, viewed in front of an ultra-violet lamp with the glass-side towards the observer and marked for any ultra-violet absorbing material if present. Finally the plate was sprayed with 10% phosphomolybdic acid or phosphotungstic acid in ethanol or 50% aqueous (V/V) sulphuric acid, heated at 110° for 10 minutes, and the colour of the spots noted. Sulphuric acid and phosphotungstic acid gave different colours with different steroids. Both these reagents gave intense blue colours with 7α or 7β-ol-5-ene and

6-ene-5α-ols. A sulphuric acid spray gave red, magenta and violet colours with 5-ene-3β-ols, and shades of yellow with saturated compounds of the 5β-series.

APPENDIX II.

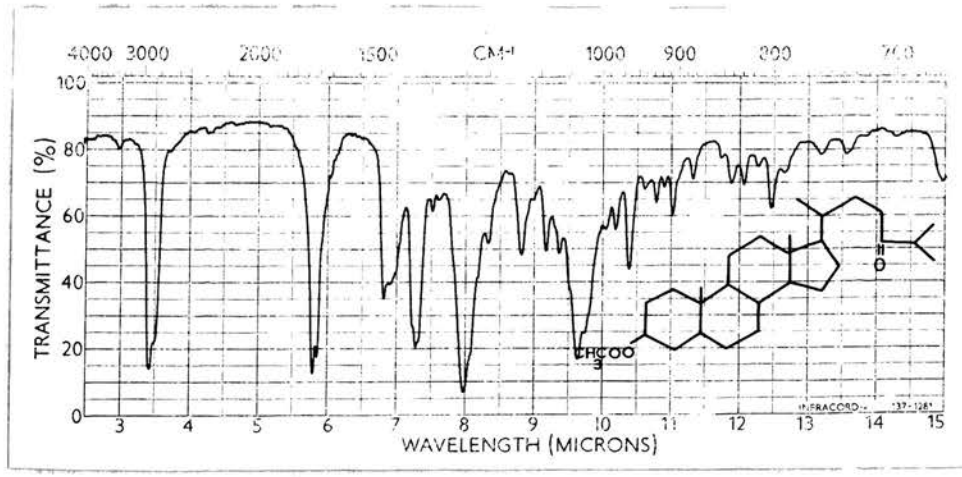


Figure. 1.

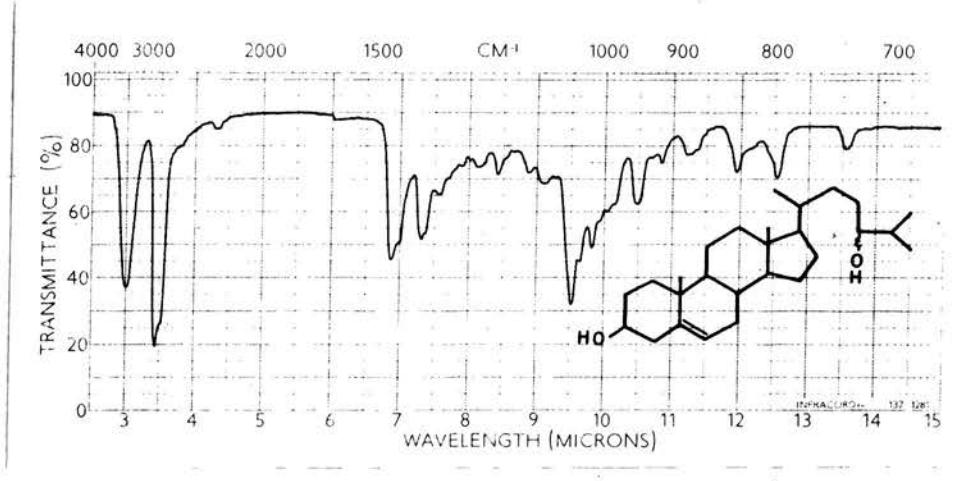


Figure 2.

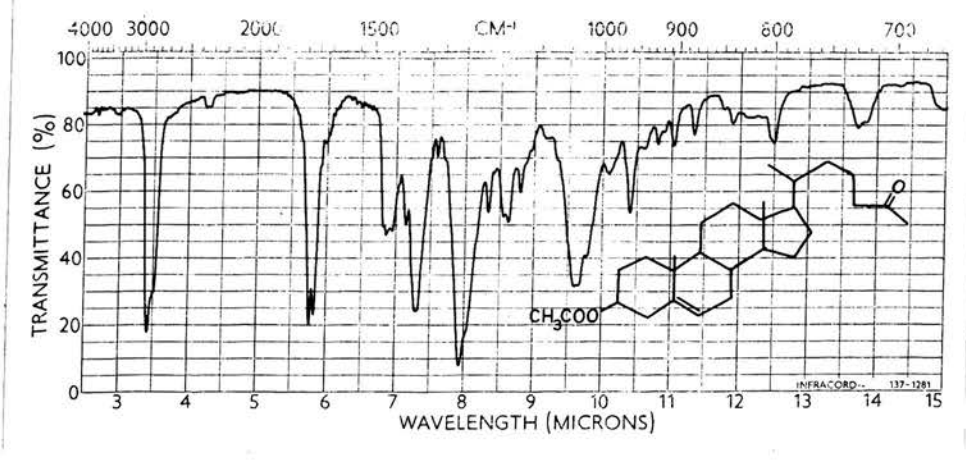
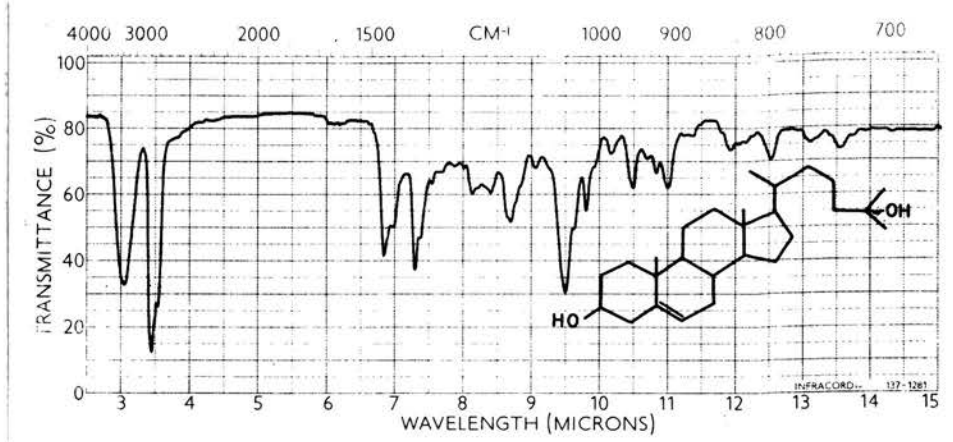


Figure 3.



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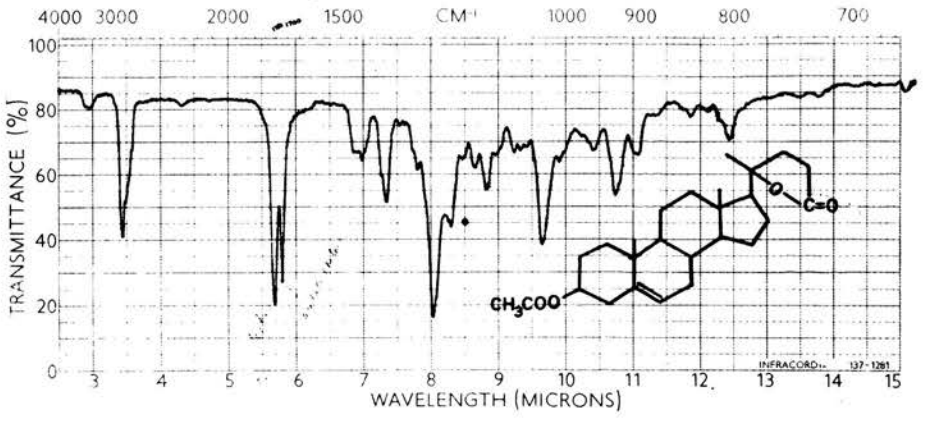

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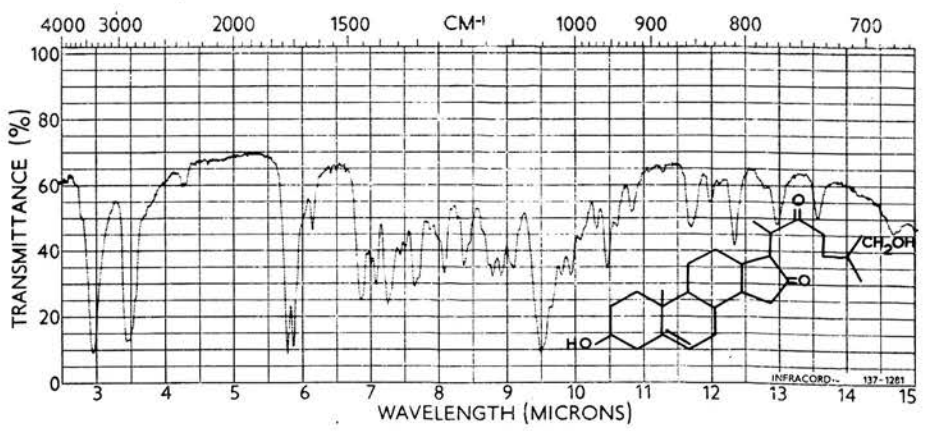

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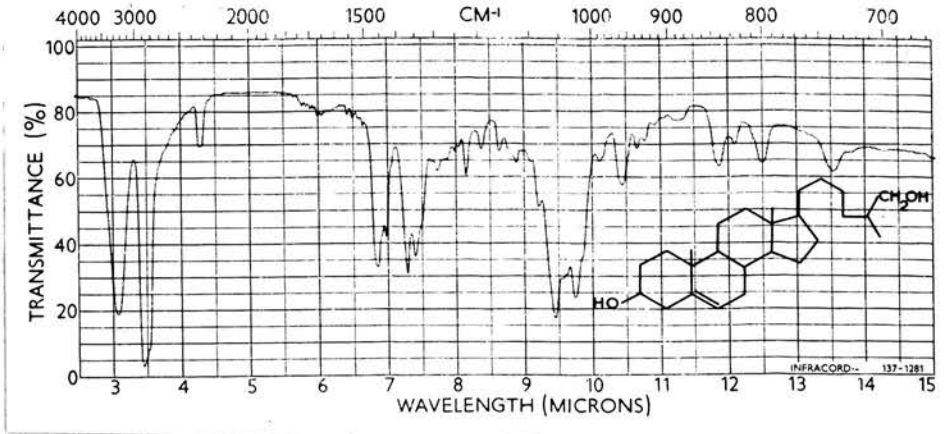

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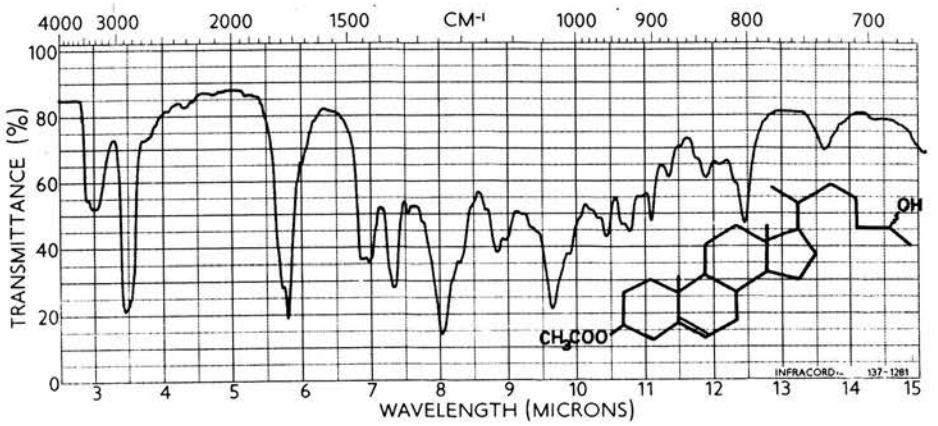

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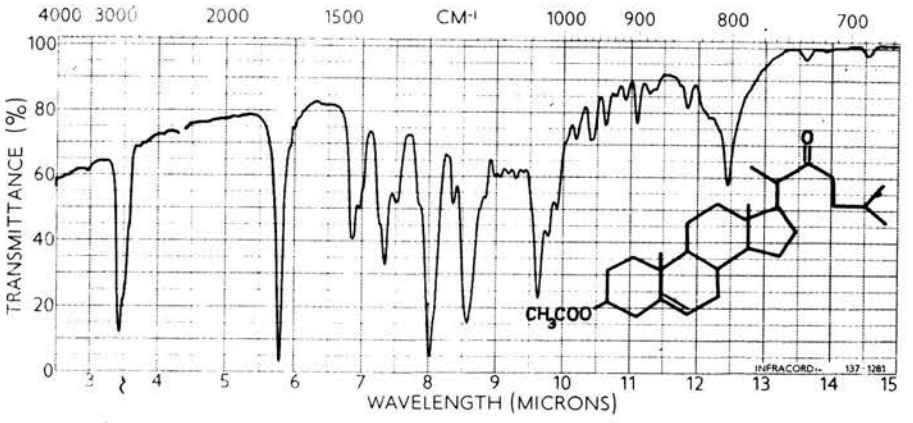

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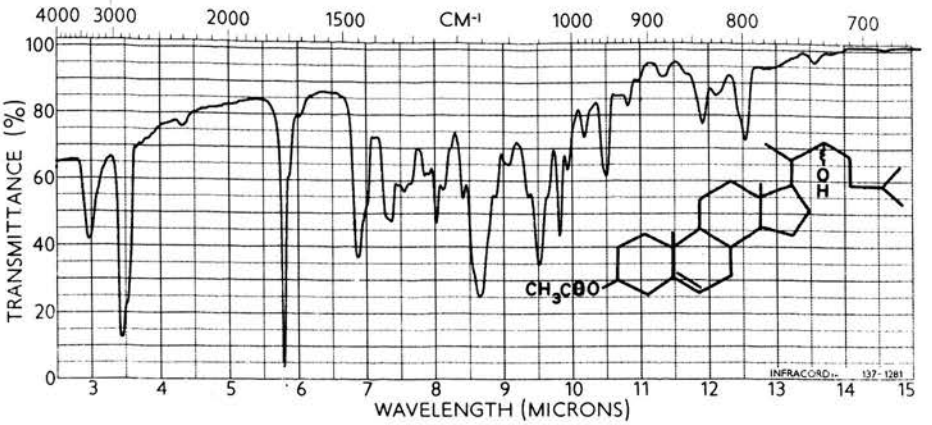

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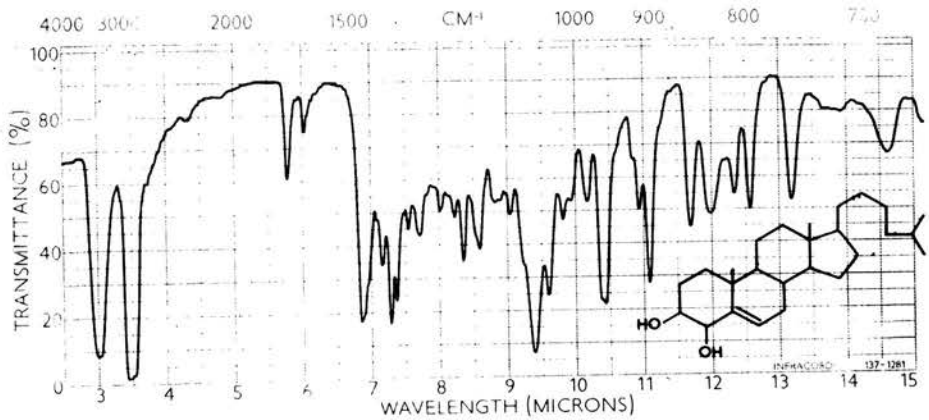

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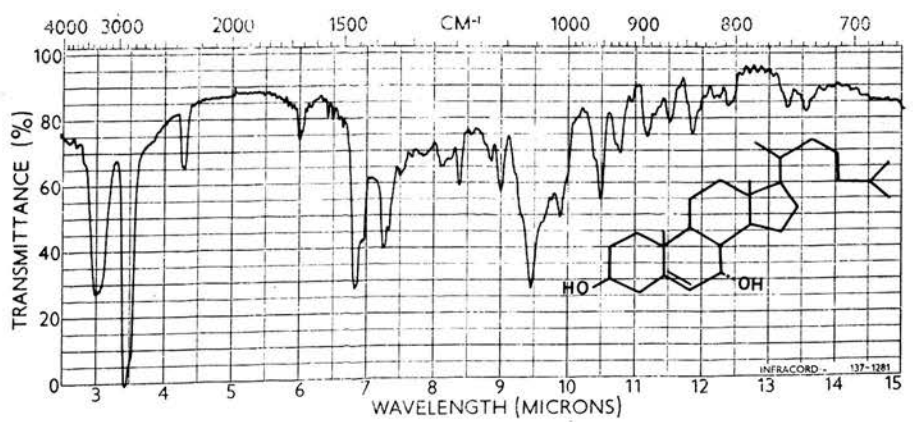

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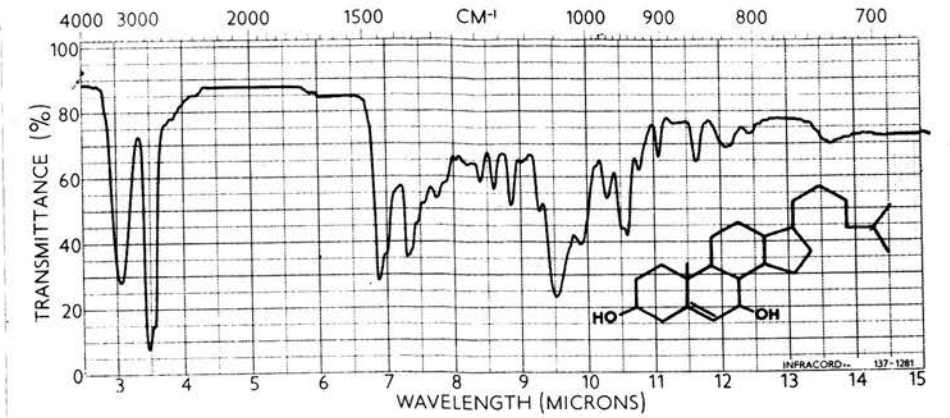

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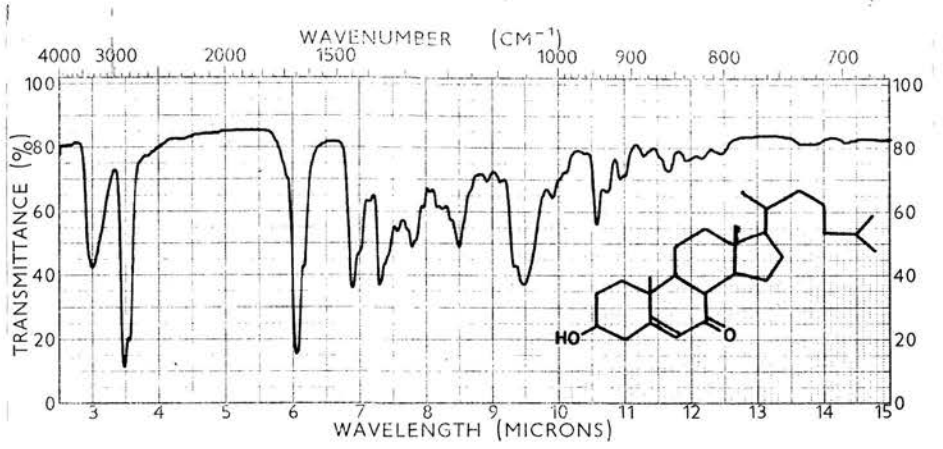

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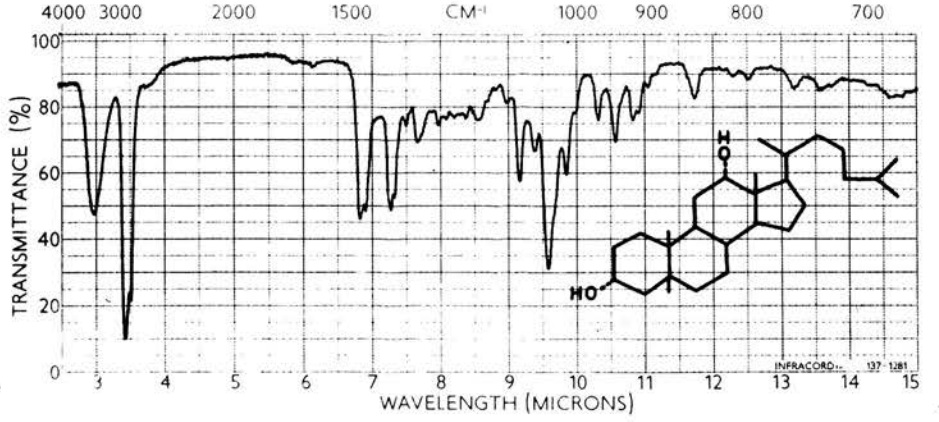

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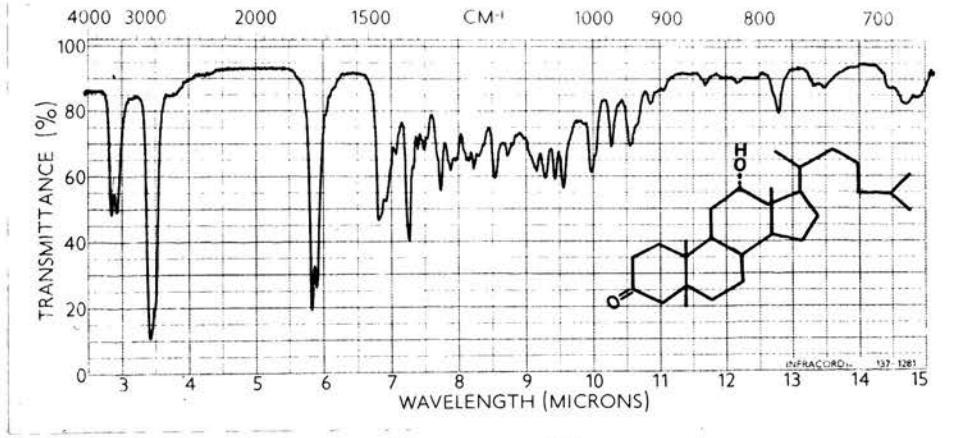

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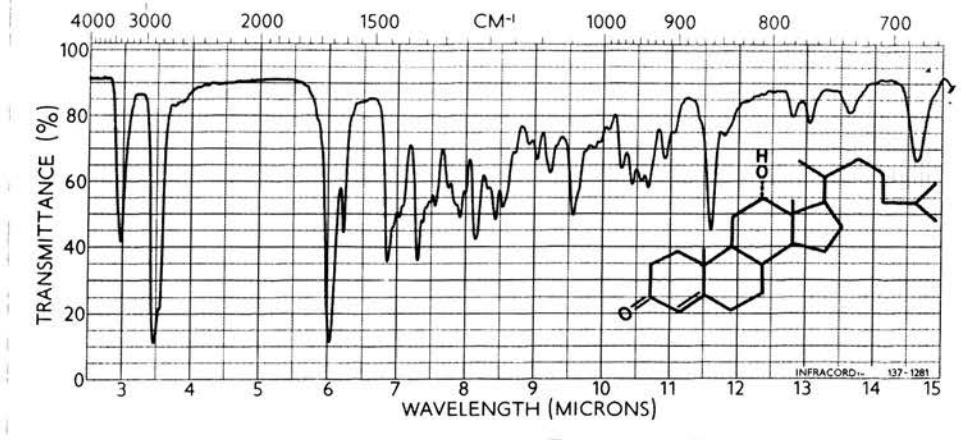

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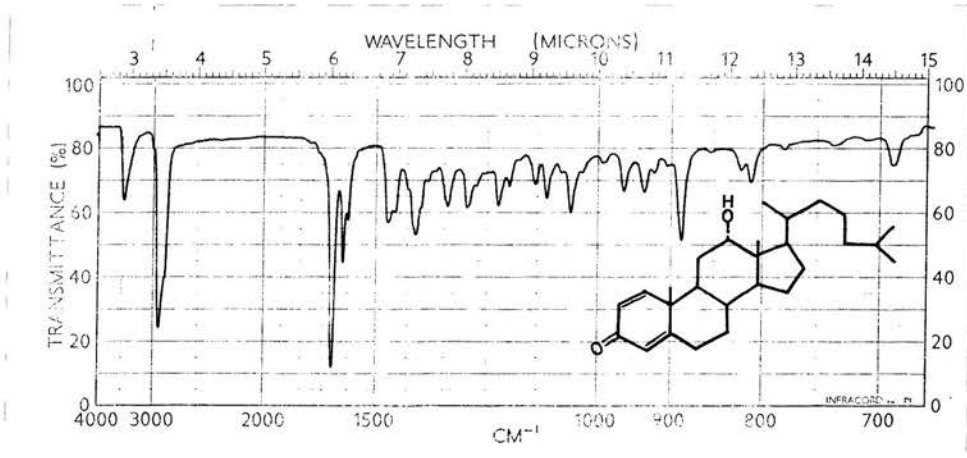

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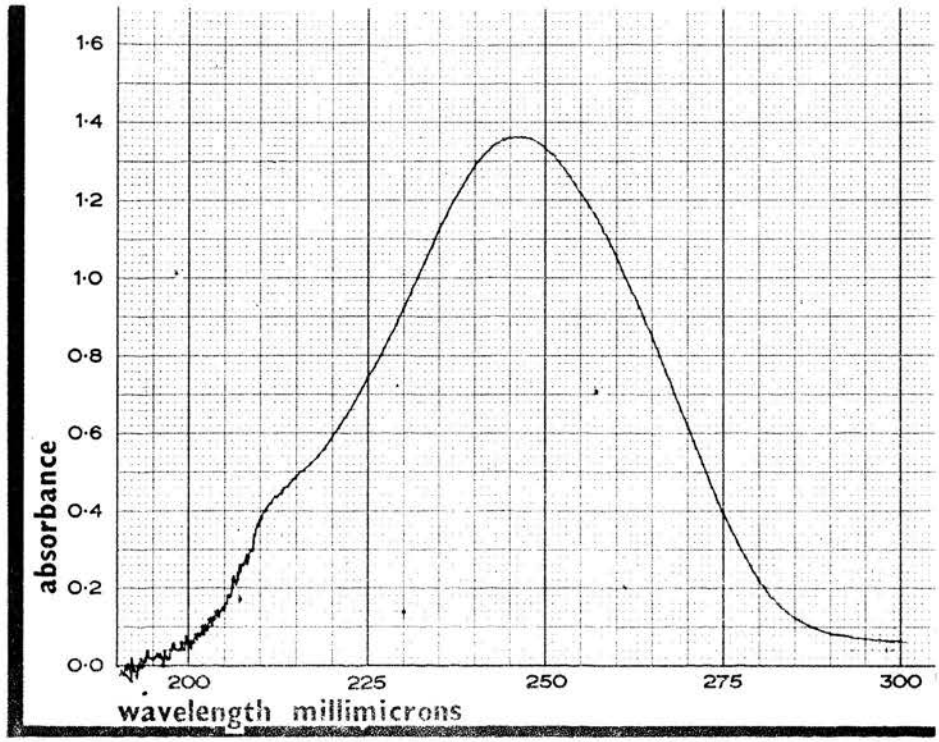
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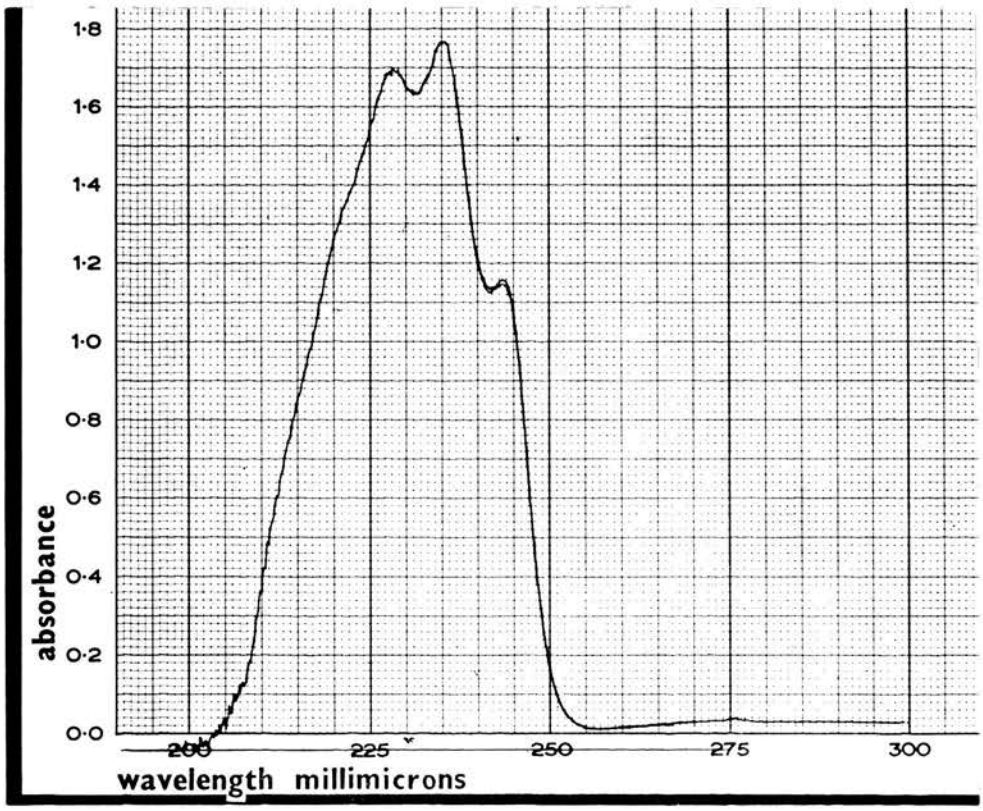
18a.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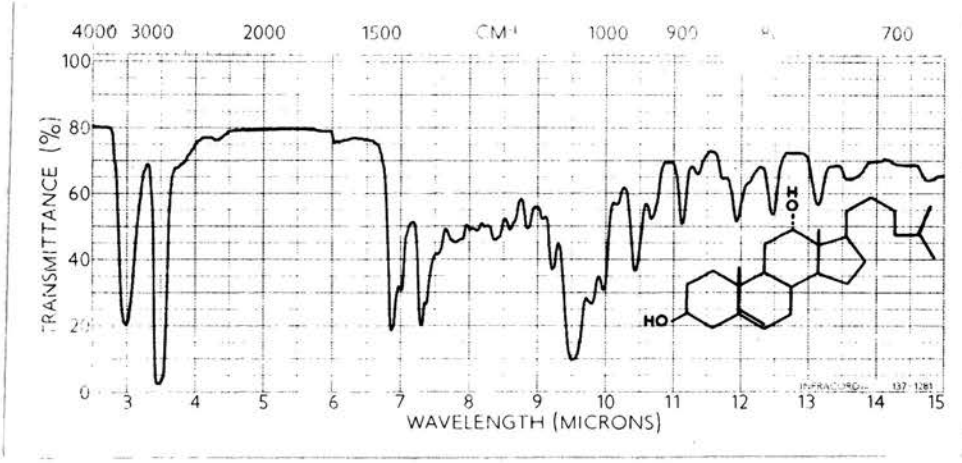

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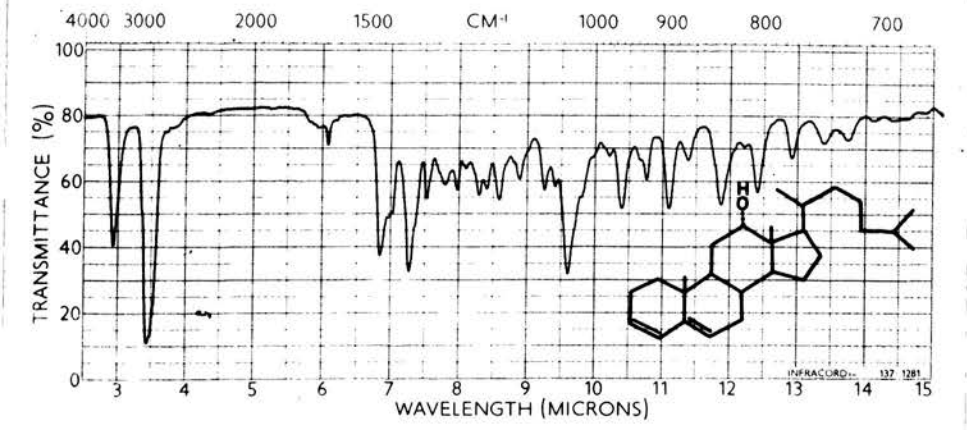

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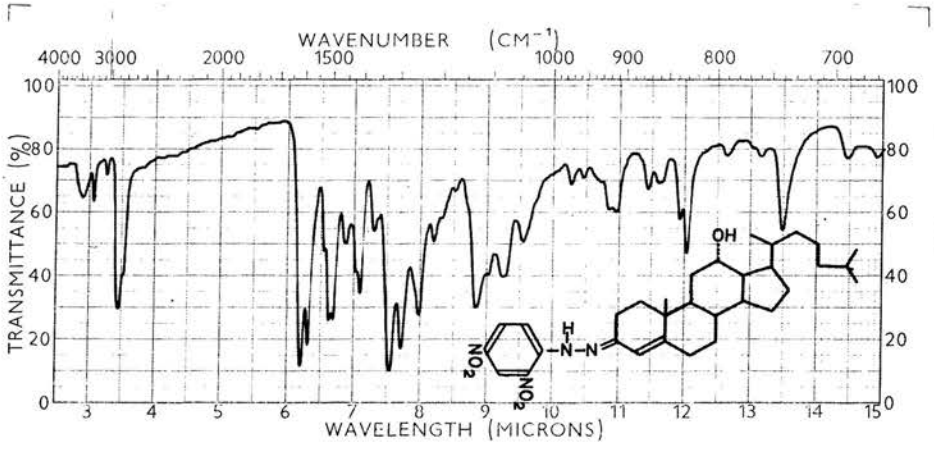

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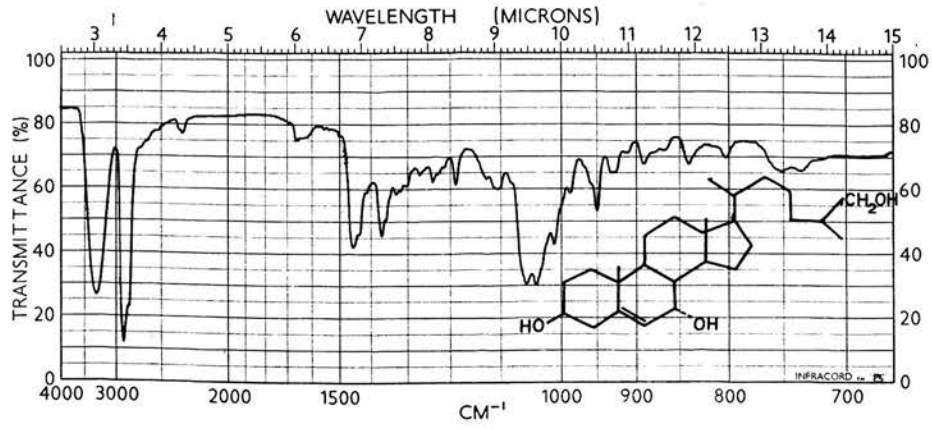

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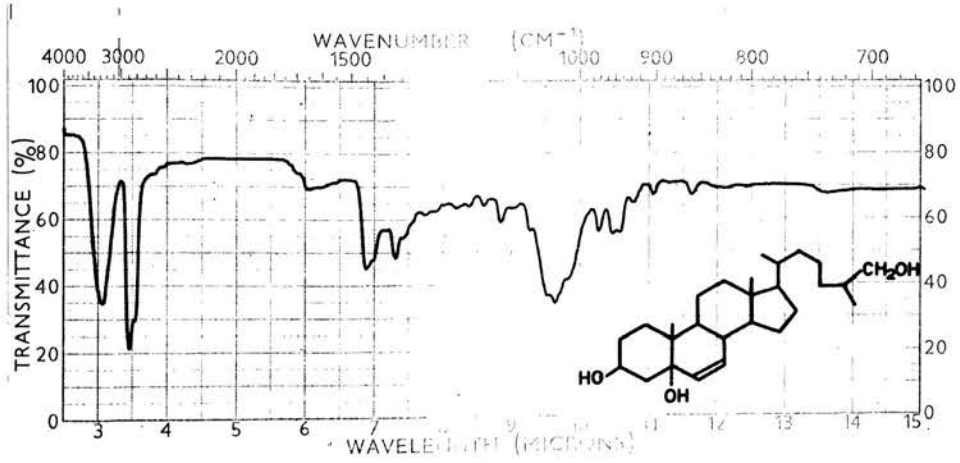

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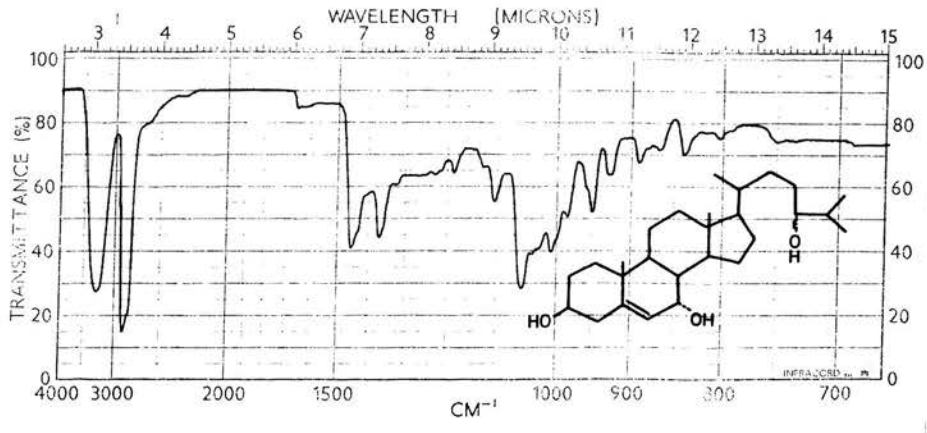

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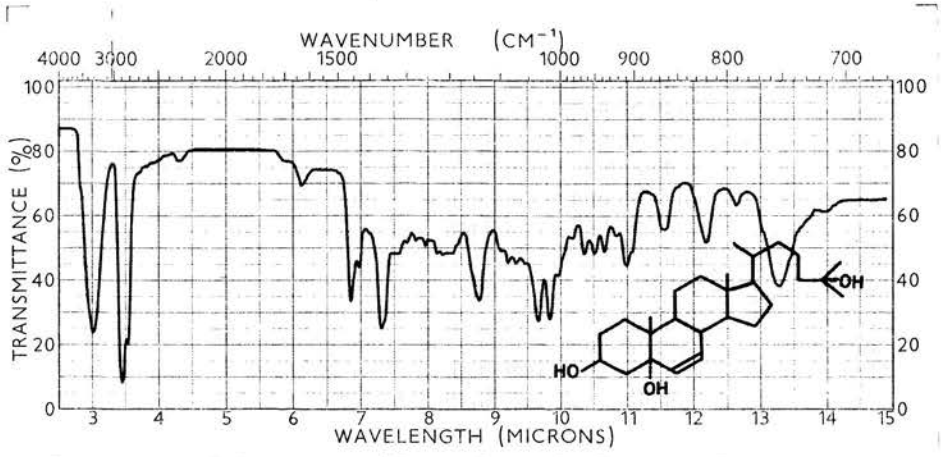

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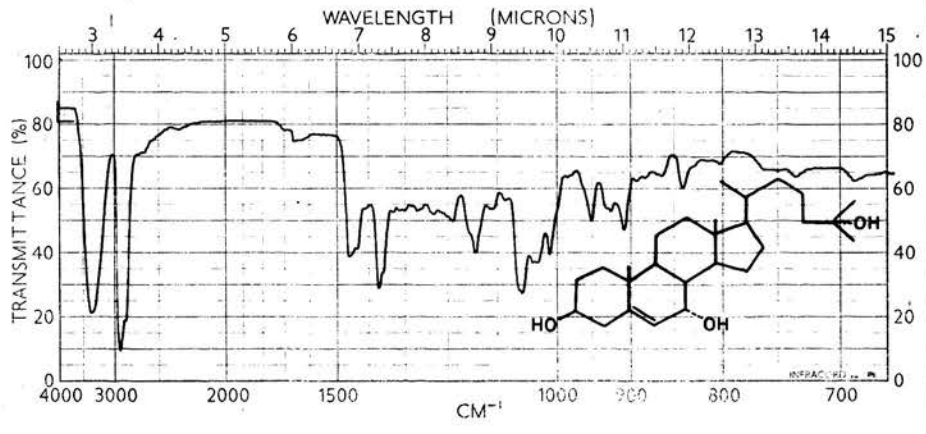

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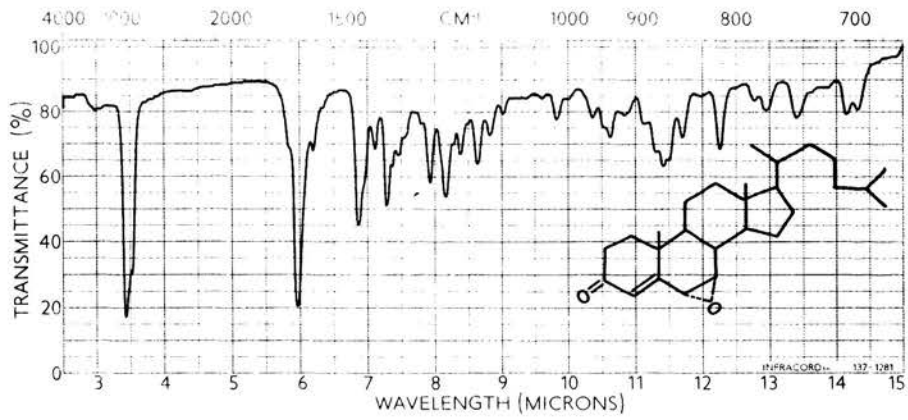

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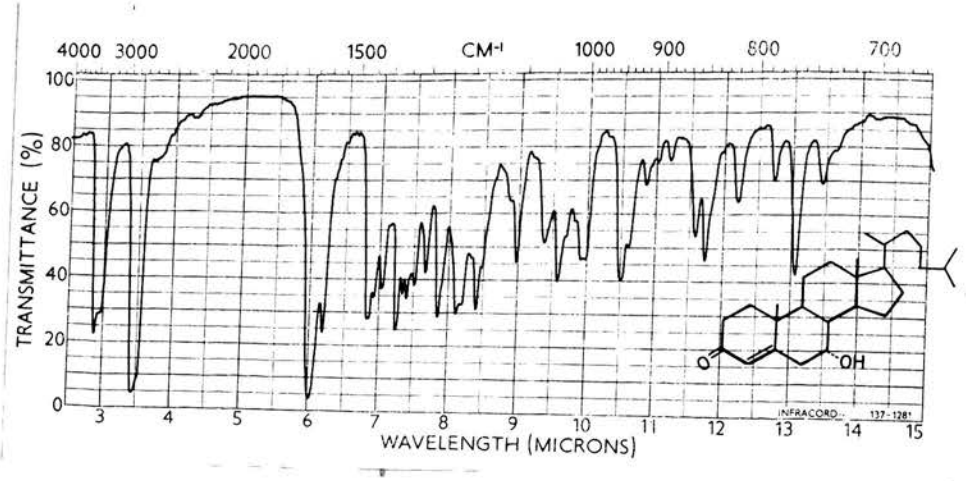

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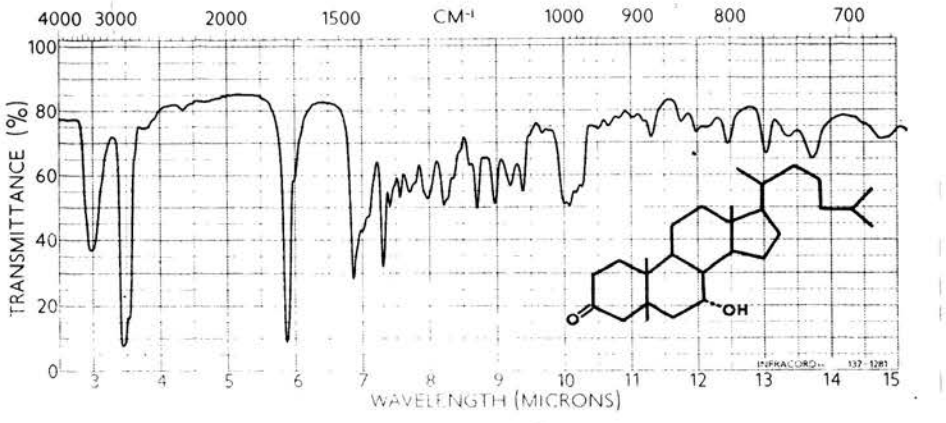

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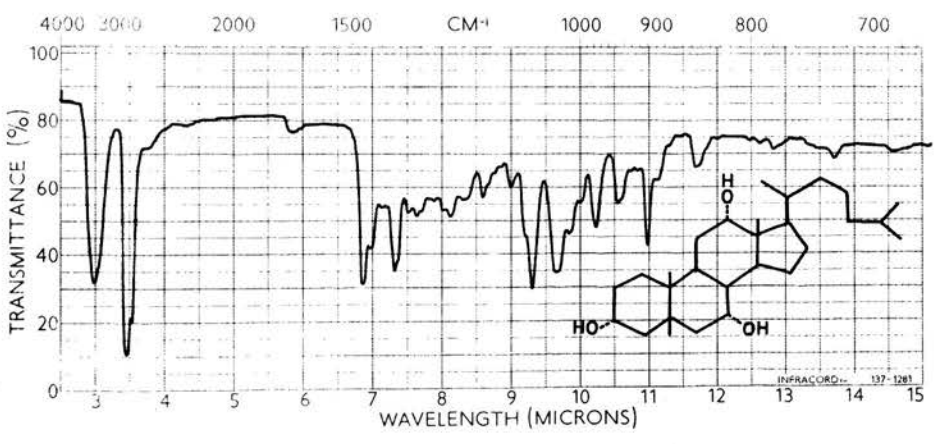

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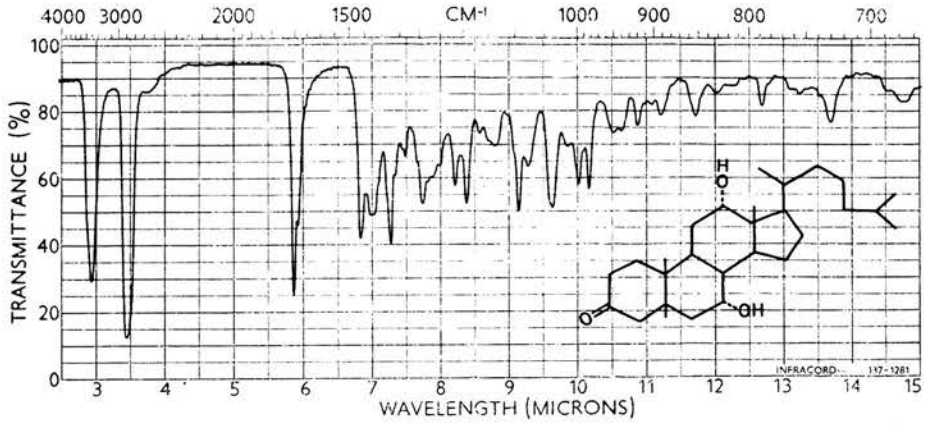

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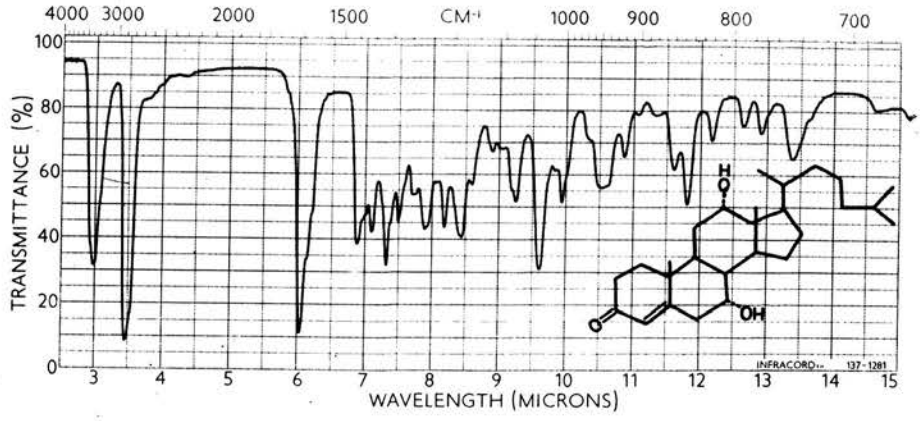

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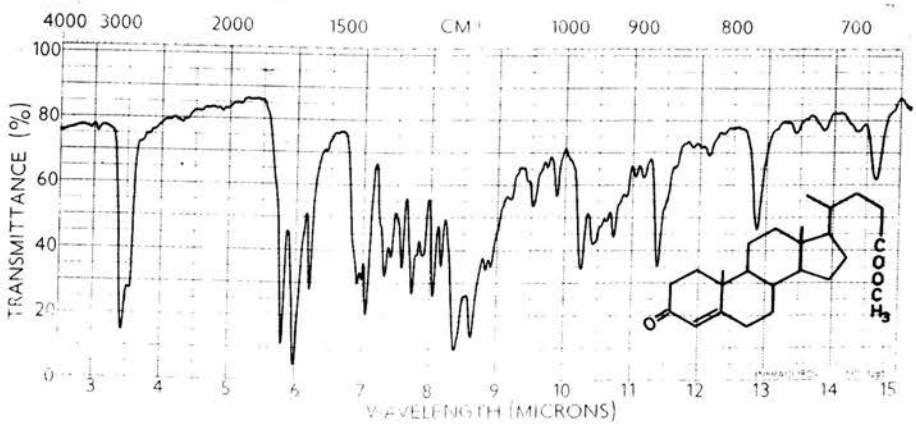

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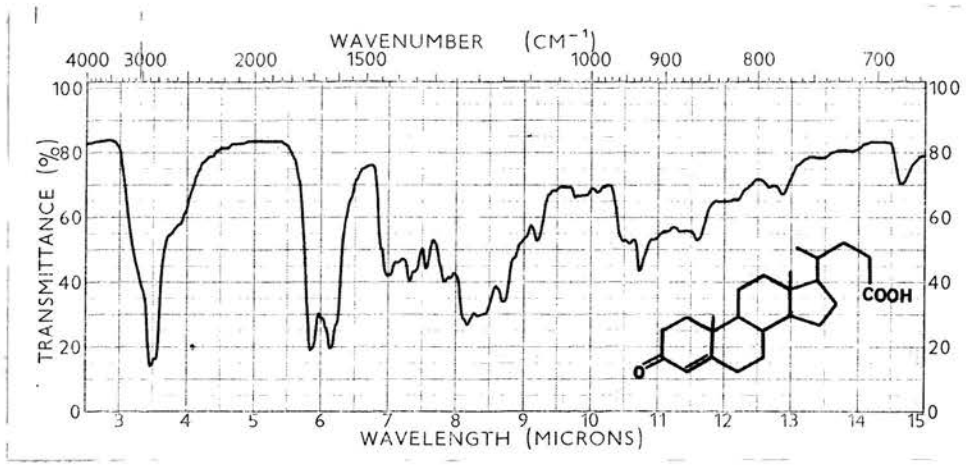

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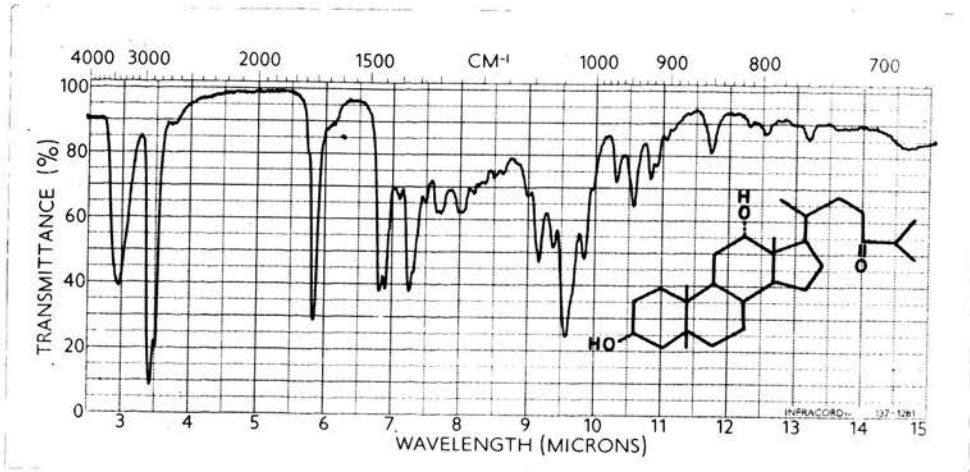

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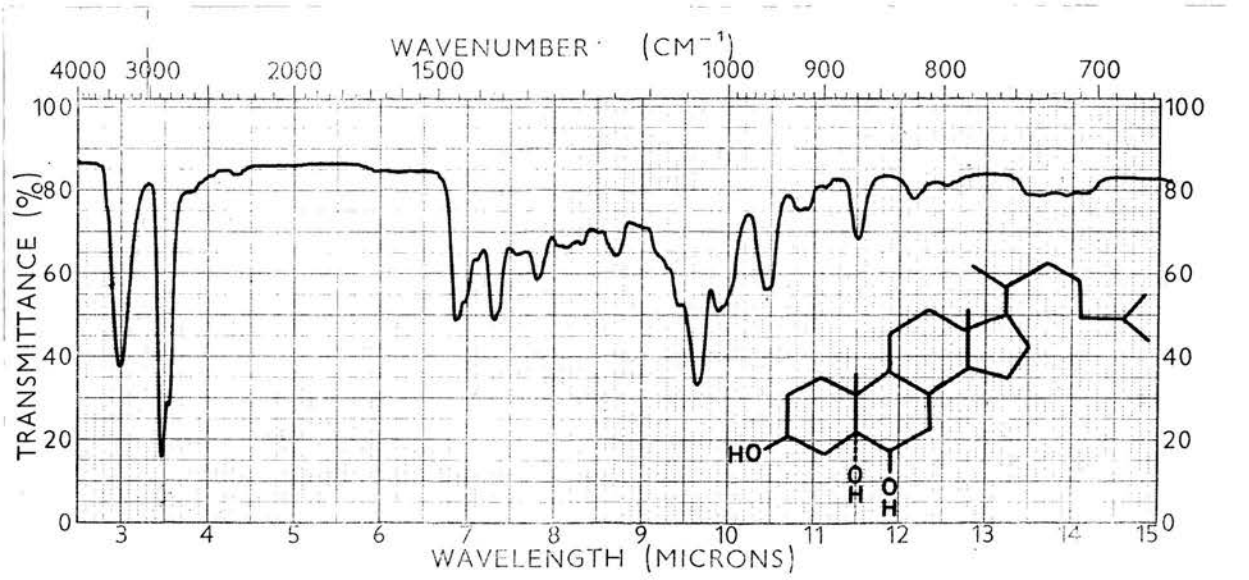

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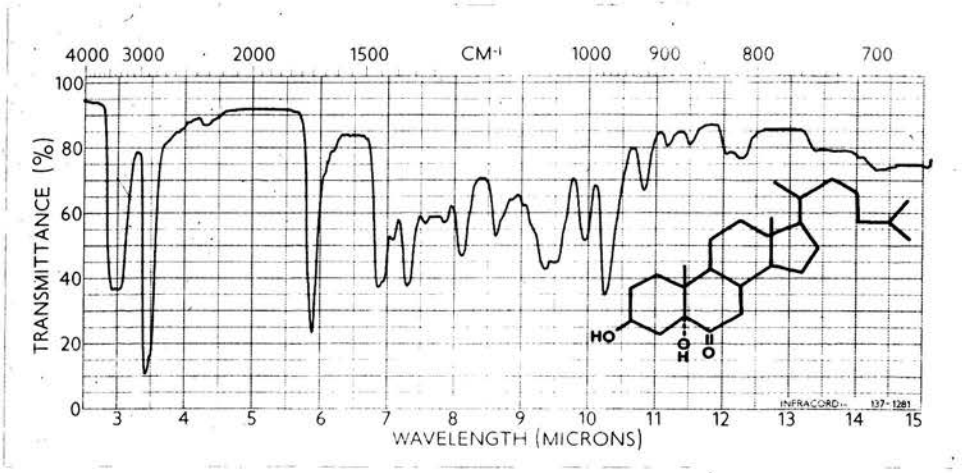

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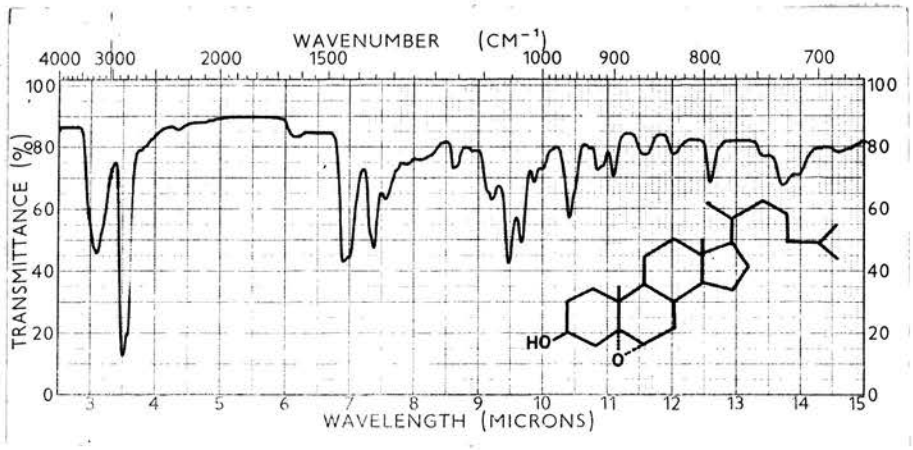

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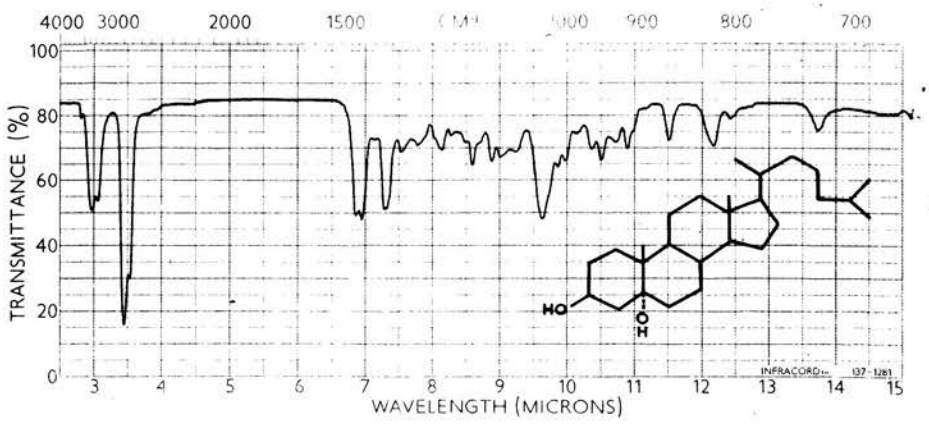

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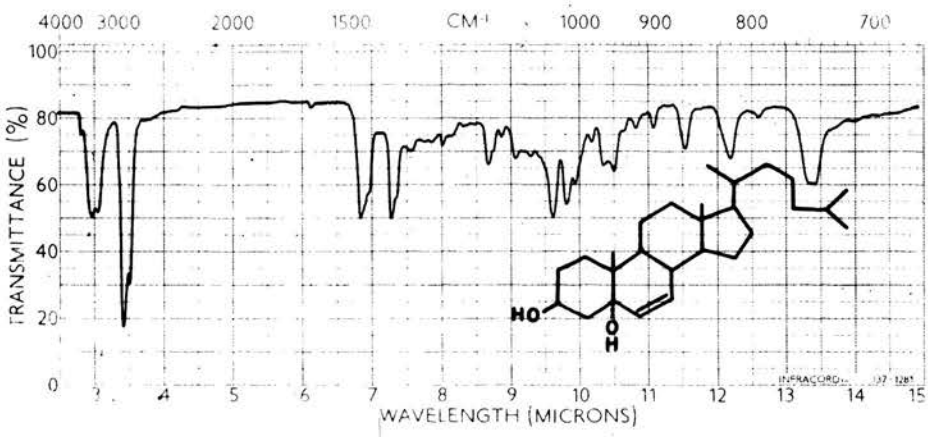

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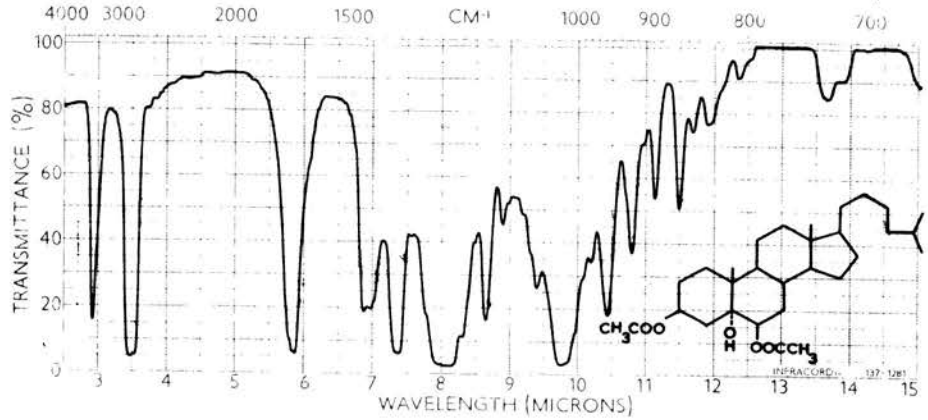

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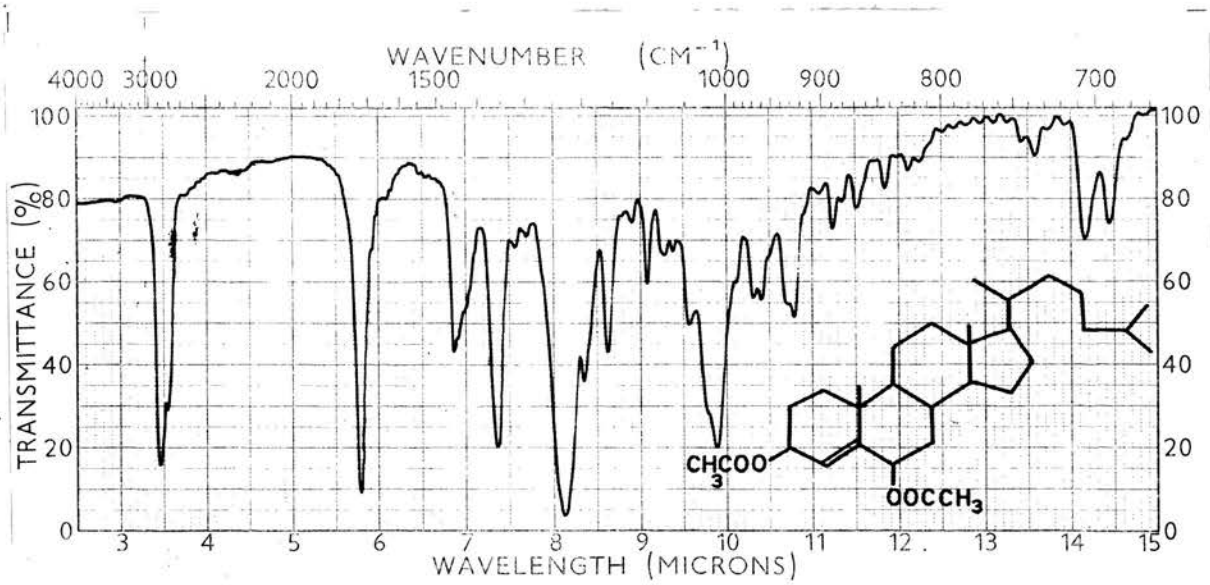

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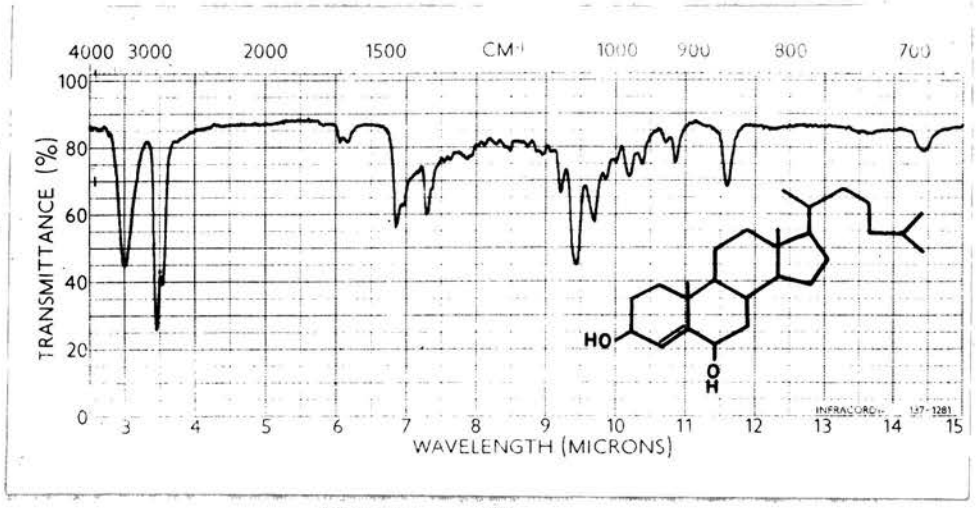

Figure 43.

Figure 44.

Figure 45.

ACKNOWLEDGEMENTS

I avail this opportunity to acknowledge my sincere gratitude to Dr. G.S. Boyd for his enthusiastic supervision and expert guidance given throughout the work reported in this thesis.

My thanks are also due to Professor R.B. Fisher for granting me admission in this department, and for his advice and encouragement.

I am also indebted to Dr. J.W. Minnis, who kindly performed elemental analysis reported herein.

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The following systematic names are given to the compounds referred to by trivial names.

Cholesterol	cholest-5-en-3β-ol
7α-hydroxycholesterol	cholest-5-en-3β,7α-diol
12α-hydroxycholesterol	cholest-5-en-3β,12α-diol
24ξ-hydroxycholesterol	cholest-5-en-3β,24ξ-diol
25-hydroxycholesterol	cholest-5-en-3β,25-diol
26-hydroxycholesterol	cholest-5-en-3β,26-diol
7-ketocholesterol	cholest-5-en-3β-ol-7-one
7β -hydroxycholesterol	cholest-5-en-3β,7β-diol
Trihydroxycoprostane	5β-cholestan-3α,7α,12α-triol
Cholic acid	3α,7α,12α-trihydroxy-5β-cholanic-acid
Chenodeoxycholic acid	3α,7α-dihydroxy-5β-cholanic-acid
Deoxycholic acid	3α,12α-dihydroxy-5β-cholanic-acid
Lithocholic acid	3α-hydroxy-5β-cholanic-acid
20α-hydroxycholesterol	cholest-5-en-3β,20α-diol
22ξ-hydroxycholesterol	cholest-5-en-3β,22ξ-diol
5β-ranol	3α,7α,12α,24,26-pentahydroxy-bis-homo-cholane
Cholenic acid	3β-hydroxy-chol-5-enoic Acid
α-Muricholic Acid	3α,6β,7α-trihydroxy-5β-cholanic Acid
β-Muricholic Acid	3α,6β,7β-trihydroxy-5β-cholanic Acid
25-keto-nor-cholesteryl acetate	cholest-5-en-3β-acetoxy-25-one